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CAN NODULE BACTERIA OF LEGUMINOUS PLANTS FIX ATMOSPHERIC NITROGEN IN THE ABSENCE OF THE HOST? [1]

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INTRODUCTION

Nitrogen-fixation studies with nodule bacteria have been reported at frequent intervals during the period since 1886–1888, when Hellriegel and Wilfarth announced that these bacteria can live in symbiosis with leguminous plants and make the growth of the latter largely independent of the nitrogen content of the soil. The findings of these two men have been verified repeatedly and are to-day practically universally accepted by research workers in this field. We know that the nodule bacteria are necessary for nitrogen fixation to take place in the leguminous plants, but we do not know the part played by these bacteria in the fixation process. The prevailing theory that the bacteria obtain carbohydrates from their host, use the energy for growth and nitrogen fixation, and in turn supply the host with nitrogen, is far from being proved. If the bacteria fix as large quantities of nitrogen as the above theory implies, then it should presumably be easy to demonstrate the fixation by the bacteria growing independent of the host. Numerous attempts have been made to do this with widely varying results. A résumé of some of the outstanding experiments along this line is given below. These are arranged for the most part in chronological order.

HISTORICAL REVIEW

Beijerinck (*3*) [3] obtained fixations varying from 0.9 to 1.8 mgm. N per 100 c. c. of media in a medium containing 5 to 7 mgm. of nitrogen. He considered that the data proved that the organism could fix nitrogen but that the increases were not greatly in excess of the errors of analysis.

Prazmowski (*32*) stated briefly, without giving data, that he had shown that the legume organism can fix nitrogen when grown on a nitrogen-free medium, but the power of fixation was not great.

Frank (*10*), in a review of his own investigations, concluded that it was very questionable whether Rhizobium had the power of fixing free nitrogen independent of the host, and was of the opinion that more likely it acted merely as a stimulus to the plant, rendering the inherent ability of the latter for nitrogen assimilation more active.

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[2] The writer wishes to thank E. B. Fred for supplying a portion of the cultures of Rhizobium used in these investigations, and for suggestions given; also F. G. Cottrell and others of the laboratory staff for their interest and help during the progress of the work.

[3] Reference is made by number (italic) to "Literature cited," p. 922.

Immendorff (*22*) was unable to secure appreciable growth or nitrogen fixation by legume bacteria growing on various nitrogen-free media. In order to secure good growths combined nitrogen was necessary in the medium.

Berthelot (*4*) grew lupine bacteria on a humic-acid medium and obtained an increase of 5.3 mgm. N in four months. For inoculation the juice from the roots of an inoculated lupine was used. Apparently a pure culture was not used, and hence the results are of little significance from the standpoint of the question under consideration.

Gonnermann (*16*) stated that the root-tubercle bacteria alone could not make the free nitrogen available for the plant, but considered that the plant itself without symbiosis could use free nitrogen.

Stutzer, Burri, and Maul (*38*) grew bacteria in 2-liter flasks, containing 50 c. c. of nitrogen-free medium absorbed in 10 gm. asbestos. After two months the analyses showed gains of 3.25, 2.23, and −0.48 mgm. N for three experiments. The authors did not consider that the results justified a definite statement as to the nitrogen-fixing power of Rhizobia.

Mazé (*26*, *27*) claimed to have demonstrated that legume bacteria can fix nitrogen if grown on a suitable organic medium, such as legume extract containing the necessary mineral elements and sugar. He was not able to secure appreciable fixation on a nitrogen-free medium. This work has been quoted so many times as proof that the legume bacteria can fix nitrogen that a critical study of the articles is very desirable. In the first place, the statements regarding nitrogen fixation are based on only about 25 or 30 analyzed cultures. The purity of these cultures was not adequately tested, and from his statements we can almost say with certainty that they were mixed. He observed a strong odor of cheese given off by the cultures and in some cases a rapid liquefaction of gelatin. These properties are certainly not characteristic of Rhizobia. He found that the bacteria decomposed sucrose very rapidly with the production of CO_2. Within 15 days usually 2 per cent sugar solutions were almost wholly converted into CO_2 with a corresponding oxygen consumption. In the first of Mazé's two articles referred to here, he reports three experiments, showing fixations of 40.8, 47.5, and 23.4 mgm. N per 100 c. c. in 15 or 16 days, starting with a medium already rich in nitrogen. In the second article the fixations in three additional experiments were 12.1, 12.8, and 15 mgm. N per 100 c. c. In the third article the fixations were −3.7, −2.4, 2.2, 0.8, 2.2, and 2.4 mgm. N in one series and 3.3, 6.0, 0.4, 2.9, and 6.3 mgm. N in another series. Most of these results were secured on media which already contained more nitrogen than the organisms would ordinarily need. The work is interesting from the historical standpoint, but in the light of our present knowledge of legume bacteria we can not seriously consider it as proof of the fact that Rhizobia can fix nitrogen independently of the host.

Greig-Smith (*17*) stated:

> Experiments were made with pure cultures of the organism, using glucose and sucrose in conjunction with plant extract, but neither with Rhizobia obtained from the lupin nor the pea could any fixation of free nitrogen be found either in faintly acid, neutral, or faintly alkaline media; the cultures finally contained the same amount of nitrogen as they had at the beginning of the experiment.

He reported also that he was unable to obtain any nitrogen fixation with nodule bacteria, grown on various media. Later (*18*) he reported numerous analyses showing some nitrogen fixation in artificial cul-

tures. In this later work he concluded that the fixation was, in general, "coincident with and proportional to the formation of slime. Under conditions that preclude the formation of slime, there is no fixation."

Neumann (*30*) obtained fixations varying from 4.4 to 49.9 mgm. N in soil and legume extracts inoculated with mixed cultures from soil, roots, and nodules, and stems and leaves. These results are of little significance from the standpoint of this discussion since pure cultures of Rhizobia were not used.

Löhnis (*25*) secured nitrogen fixation on a soil-extract medium, the quantities varying from 0 to 1.54 mgm. N per 100 c. c. of medium in 21 days.

Moore (*28*) obtained fixations of 0.2 to 2.2 mgm. N where various strains of legume bacteria were grown in an aerated nitrogen-free medium. The nitrogen was found to be stored up within the bacterial cells as protein and could not be removed by merely filtering off the medium.

Lewis and Nicholson (*24*) report fixations with alfalfa bacteria varying from 0 to 16.2 mgm. N per 100 c. c. of media, with an average of approximately 2.2 mgm. N. The results appear to be rather erratic, the largest fixations being secured in media which contained far more nitrogen than the bacteria would be expected to assimilate under the conditions.

The work of Golding (*15*) has probably been more widely quoted in recent years as proof that nodule bacteria can fix nitrogen than that of any other worker. It has been repeatedly stated in the literature that he demonstrated that by the continued removal of the soluble products of growth from bacterial cultures they fixed comparatively large quantities of nitrogen. Let us see what Golding, himself, said. In his first experiment a gain of 342 mgm. N was obtained where 520 gm. of macerated legume stems, leaves, and nodules were present in 3,000 c. c. of medium, which was constantly being removed by suction. This medium was not sterilized and hence the results mean nothing so far as demonstrating that legume bacteria can fix nitrogen. In a second experiment 500 c. c. of fresh unheated cold-water extract of pea plants with the addition of 5 gm. of dextrose served as a medium. In this impure culture the fixation was 24.4 mgm. N, again showing nothing regarding the ability of root-nodule bacteria to fix nitrogen, because pure cultures were not used. In a third experiment, using a synthetic nutrient medium containing 1,550 c. c. of sterile pea extract inoculated with a pure culture, a fixation of 31.5 mgm. N was obtained in 13 days. In a similar pure-culture experiment, starting with 250 c. c. of sterile plant-extract medium, the fixation was 7 mgm. in 22 days. The quantity of nitrogen fixed was, therefore, 2.0 and 2.8 mgm. N per 100 c. c. of medium, respectively, for the two pure-culture experiments. This fixation is no greater than that reported by certain other investigators as occurring in ordinary culture flasks and is not greatly in excess of experimental error, considering the rather difficult technic used and the fact that plant extracts were employed. It is very difficult to secure accurate analyses with such material, and even if closely agreeing duplicate determinations are obtained there is no guarantee that the analyses are really accounting for every form of nitrogen present. The recent paper of Christensen (*9*) is of interest in this connection. It is also worth while to empha-

size that Golding like Mazé obtained his fixations with Rhizobium grown on a medium already containing more nitrogen than the bacteria needed. Certainly, numerous repetitions of the work would be necessary to make the results really conclusive. Golding tried various other methods to demonstrate fixation in pure cultures, but without success. Results were unsatisfactory where agar cultures were floated on sugar solutions in sterile parchment dialyzers; likewise, where cultures were grown in flasks in thin layers; and again where sugar was added to the cultures daily.

Fred (*11*), using 15 strains of legume bacteria, reported fixations varying from 17 to 34 mgm. N per 200 c. c. of a nitrogen-deficient medium. However, in a private communication he states that there was an error in printing and that the data should be based on 1,000 c. c. instead of 200 c. c. of media. The average fixation was, therefore, about 1.5 mgm. N per 1,000 c. c. In a second paper (*12*) he reported fixation for several strains of nodule bacteria grown in liquid, sand, and soil cultures. The approximate average nitrogen gain for the pure cultures was 8.9 mgm. N per 1,000 c. c. of liquid media, 8.5 mgm. N per 100 gm. sand, and 5.2 mgm. N per 100 gm. clay soil. The author summarized the work as follows:

> While the nitrogen assimilation in the liquid medium, in the sand, and in the soil, was very small, it was sufficient to give a reasonable basis for the belief that *Bacillus radicicola* will live in the soil without the host plant and accomplish a certain amount of nitrogen assimilation.

In a later report (*13*) of a rather extensive series of experiments, he obtained fixations of usually less than 1 mgm. N per 100 c. c. with a few increases as high as 3 mgm. N. The results were usually higher on agar cultures than in solution. The presence of a trace of nitrogen seemed to aid in the gain, while large amounts retarded the process. He stated that the bacteria fixed as much, or more, nitrogen per unit of carbohydrate consumed than such a form as Azotobacter. In a bulletin by Fred, Whiting, and Hastings (*14*) fixations of 14.15 and 18.55 mgm. N were obtained per 15,000 c. c. of media in two experiments where legume bacteria were grown on Berkefeld filters with provision for the constant addition of fresh medium and removal of the old. Most of the nitrogen assimilated was held in the residue on the candle and only a very small part was carried into the filtrate.

Rossi (*34*) made a study of the nitrogen-fixing power of legume-nodule bacteria grown on gelatin, agar, and liquid media and obtained negative results. An experiment conducted somewhat along the lines of Golding's (*15*) work also showed no significant gain in nitrogen.

Buchanan (*8*) reported the results of a detailed study of the gum produced by *Bacillus radicicola* and stated that it contained no combined nitrogen.

Bottomley (*5*) secured a fixation of 2.3 mgm. N per 100 c. c. of synthetic nitrogen-free medium with nodule bacteria. He repeated the work later (*6*), making the analyses after all sugar was used up, and secured similar results. In 1912 (*7*) he isolated the bacteria from the root nodules of *Myrica gale* and found them identical in structure and growth with the legume nodule bacteria. In liquid cultures a fixation of 2.05 mgm. N per 100 c. c. was obtained in seven days.

Spratt (*37*) also isolated bacteria from the nodules of nonlegumes, Podocarpinae, found them identical with *Pseudomonas radicicola* obtained from the Leguminosae, and secured a gain of 2.5 mgm. N

by the organism grown in pure culture. In another paper (*36*) she reports fixations of 2.5 to 3.5 mgm. N by *P. radicicola* isolated from the root nodules of Alnus and Elaeagnus and grown in pure culture in laboratory media.

Herke (*20*) determined the nitrogen-fixing power of legume bacteria and concluded that a soil extract medium favors fixation, the rate being an average of about 0.4 mgm. N per 100 c. c. of medium in unaerated cultures. Increasing aeration, either by the use of thin layers of media or by bubbling air through the cultures, slightly increased fixation. Where the action of the plant was imitated by growing the organisms in funnels on kieselguhr with the periodic addition of mannit solution and removal of the old medium by suction, the fixation varied from 1.19 to 3.08 mgm. N per flask.

Olaru (*31*) reported fixations varying from 1.5 to 32.1 mgm. N per 100 c. c. of media in plant extracts containing 2 per cent sucrose and varying percentages of $MnSO_4$, inoculated with pea-nodule bacteria.

Mulvania (*29*) found that where a dialyzing membrane intervened between a legume bacterial culture and the roots of the host plant no infection of the roots occurred and the plants did not benefit by the presence of the bacteria, showing that no usable dialyzable nitrogen was produced by the bacteria.

Rocaolano (*33*), starting with a medium containing 28.9 mgm. N per 100 c. c., added $MnCl_2$ in amounts varying from 0.001 to 0.02 gm. of Mn ion per 100 c. c. of medium. The nitrogen fixation by the nodule bacteria increased from 3.1 mgm. in the control to 9.6 mgm. with 0.006 gm. of Mn ion per 100 c. c. With higher concentrations the fixation decreased markedly.

Hills (*21*) claimed to have demonstrated that Rhizobia fixed a small quantity of nitrogen, varying from 0.15 to 0.43 mgm. N per 100 c. c. of media, when grown on agar films. He claimed, further, that the addition of nitrates increased the fixation slightly, the increases under these conditions varying from about 0.53 mgm. to 3.5 mgm. N per 100 c. c. A critical study of the data shows, however, that the method of analysis used accounted for less than half of the nitrogen actually added to the cultures as nitrate.

Joshi (*23*) inoculated three strains of nodule bacteria separately into soil-extract-mannit media and determined the nitrogen fixation after 10 days. The increase in nitrogen, due to the bacteria, varied from 0.8 to 1.2 mgm. N per 100 c. c. Several additional strains gave an average fixation per 100 c. c. of 1.4 mgm. N with a maximum of 2 mgm. N. No details of the analytical methods are given and only a few figures.

Barthel (*2*) attempted to establish definitely whether leguminous nodule bacteria possess the faculty of fixing nitrogen, when grown in pure cultures, and obtained negative results with a culture isolated from the pea.

Halversen (*19, p. 400*) reported fixations of 1.2 and 1.7 mgm. N by *Rhizobium leguminosarum* grown for eight days on synthetic media. He states that the amounts of nitrogen fixed "are so small for the Rhizobium cultures that the experimental error is often greater than the amount of the determination." In one case, however, he obtained a fixation of 7.2 mgm. N in a culture containing lime, which was aerated for four weeks.

Skinner (*35*) obtained no fixation with alfalfa bacteria and reports that the finding was entirely unexpected.

The writer (*1*) in a preliminary statement reported that red-clover bacteria failed to fix nitrogen when grown on plant-extract media.

A study of the investigations discussed above emphasizes certain outstanding points: (1) Rarely has Rhizobium been reported as making a good growth or showing fixation appreciably above the experimental error when grown on a nitrogen-free medium. (2) Where small amounts of nitrogen were present, particularly in miscellaneous unknown forms such as soil extract, gains of nitrogen have been frequently reported but these increases have usually been less than 1 mgm. It should also be borne in mind that under the same conditions in a number of instances no increases of nitrogen have been secured and a number of losses have been recorded. (3) When large quantities of nitrogen were present in the medium at the start the results have been very much more erratic, showing in some cases marked losses and in others increases up to about 50 mgm. N. Fairly large fixations of nitrogen have frequently been reported for legume-nodule bacteria grown in plant-extract media. Usually where the largest increases were obtained the bacteria were grown in media that contained from two to five times as much nitrogen as the bacteria would need for a normal growth, assuming no fixation. It is true that not all of the nitrogen in media, such as plant extracts, is available, but certainly a good percentage is. A further important fact brought out by a careful study of the references given is that the conclusions in most instances have been based upon a very limited amount of data and very few extensive studies of the question have been reported. Much of the earlier work is questionable because very obviously pure cultures were not always used.

EXPERIMENTAL WORK

In an attempt to clarify the situation a rather extensive series of experiments was initiated at this laboratory in May, 1923, and these have been continued to the date of writing, December, 1928. Most of the work reported in this paper was completed more than two years ago but not published because of the desire to test thoroughly the numerous suggestions that have been given in the literature and by personal contacts. Because of the fact that the results reported below are of the negative type a logical presentation is impossible. The experimental work consisted mostly in testing the nodule bacteria for nitrogen fixation under as many different conditions as possible. Failing in one experiment to obtain nitrogen fixation, other experiments were started which frequently had little bearing on those previously conducted, except that they may have been designed to bring some additional factor or factors into the picture. These heterogeneous experiments are, therefore, grouped together and presented below.

METHODS

Many schemes were tested by the writer for the growth of Rhizobia in special types of apparatus designed to favor fixation, but in most instances the organisms were grown at 28° C. in 100 c. c. portions of

media contained in 300 c. c. Erlenmeyer flasks, and unless otherwise stated, it may be assumed that this was the procedure used in all experiments reported here. In many cases flasks having capacities up to 3 liters were used in order to provide greater aeration. The composition of the medium was the chief variable. As a basis for most of the media, Ashby's solution with slight modifications was chosen. When the term "Ashby's solution" is used in the following pages, a solution of the following composition is meant:

	Grams		Grams
Magnesium sulphate	0. 2	Ferric chloride	0. 0005
Dipotassium phosphate	. 2	Calcium carbonate	1. 0
Sodium chloride	. 2	Water	1, 000. 0
Calcium sulphate	. 1		

The sugar used was usually 1 per cent dextrose, but this varied and reference must be made to each individual experiment. To this medium as a basis various additions were made and their influence, if any, on nitrogen fixation determined.

Nitrogen determinations, unless otherwise stated, were made by the Kjeldahl method, using $CuSO_4$, K_2SO_4, and H_2SO_4 for digestion. In some of the experiments, reported near the end of this paper, analyses were made by the Kjeldahl method, using metallic mercury and H_2SO_4 for digestion, followed by precipitation of the mercury with sodium thiosulphate prior to distillation.

NITROGEN-FIXATION STUDIES ON NITROGEN-FREE MEDIA

The usual method of testing a microorganism for nitrogen-fixing power is to grow it in pure culture in a liquid, or in some cases solid, medium containing the necessary inorganic elements for its growth and a suitable carbohydrate. Ordinarily organisms show their highest rates of fixation on such a nitrogen-free medium or at least on a medium containing only a comparatively small quantity of nitrogen. As the supply of available nitrogen is increased appreciably fixation decreases. In other words the microorganism uses atmospheric nitrogen only when it is forced to do so and prefers the simple nitrogenous salts.

The first experiment started, and much of the later work, was based on this idea that in order to demonstrate nitrogen fixation the legume bacteria should be grown under starvation conditions, so far as fixed nitrogen is concerned. In this first experiment two media were chosen, Ashby's solution with the addition of 1 per cent mannit and Hunter's medium, consisting of magnesium sulphate 0.2 gm., dipotassium phosphate 0.5 gm., sodium chloride 0.2 gm., and mannit 10 gm. per liter, adjusted to pH 7.0 to 7.4. Cultures were grown both with and without $CaCO_3$. A portion of the flasks were inoculated with soybean bacteria and others with a culture of *Azotobacter vinelandii* obtained from the New Jersey Agricultural Experiment Station. Optimum growth conditions for the two organisms are similar and hence the use of Azotobacter served as a check on the soybean culture. Table 1 gives the analyses after 16 and 24 day growth periods at room temperatures.

TABLE 1.—*Results of nitrogen-fixation tests with soybean bacteria and Azotobacter vinelandii grown on two different media and incubated at room temperature*

ASHBY'S MEDIUM

Culture	Incubation period	With calcium carbonate				Without calcium carbonate			
		Growth observations	N	Average	N fixed	Growth observations	N	Average	N fixed
	Days		*Mgm.*	*Mgm.*	*Mgm.*		*Mgm.*	*Mgm.*	*Mgm.*
Control, uninoculated.	16		0.0 0	0					
Soybean bacteria	16	Slight	0 .04	.02	0.02	Slight	−0.03 0	−0.02	−0.02
A. vinelandii	16	Good	Lost. 6.23	6.23	6.23	Good	6.62 5.34	5.98	5.98
Soybean bacteria	24	Slight	−.11 .03	−.04	−.04	Slight	−.17 −.04	−.11	−.11
A. vinelandii	24	Good	5.17 4.79	4.98	4.98	Good	5.63 5.36	5.50	5.50

HUNTER'S MEDIUM

Soybean bacteria	16	Slight	0.14 Lost.	0.14	0.14	Slight	0.11 .0	0.06	0.06
A. vinelandii	16	Good	5.45 5.84	5.65	5.65	Fair	3.11 3.70	3.41	3.41
Soybean bacteria	24	Slight	−.01 −.04	−.03	−.03	Slight	−.01 .13	.06	.06
A. vinelandii	24	Good	5.11 4.85	4.98	4.98	Fair	3.19 3.45	3.32	3.32

It will be observed that the soybean organisms did not fix nitrogen in either of the two media used whether in the presence or absence of calcium carbonate. The results in all cases are wholly within the experimental error. So far as growth of the soybean bacteria was concerned the observations checked with the analysis, about the only evidence of growth being a slight amount of gum formation.

The culture of *Azotobacter vinelandii* fixed nitrogen under all conditions, the quantity being greatest in Ashby's medium containing calcium carbonate and lowest in Hunter's medium without calcium carbonate. It will be observed, further, that in every instance the quantity of nitrogen found in the flasks inoculated with Azotobacter was greater at the end of 16 than of 24 days. This was probably due to the loss of a volatile form of nitrogen from such cultures.

NITROGEN-FIXATION STUDIES WITH VARIOUS STRAINS OF NODULE BACTERIA

Another experiment, similar to that reported in Table 1, was started soon after its completion for the purpose of testing cultures from a large number of species of legumes. The experimental methods were, in general, the same except that the temperature of incubation was 25° C. and a soil-extract medium, prepared according to the recommendations of Löhnis, was substituted for Hunter's medium. This contained in addition to the extract, 0.5 gm. dipotassium phosphate, 10 gm. mannit, and excess of calcium carbonate in a liter of water. The Ashby's solution used contained 20 gm. of mannit per liter. The bacterial cultures used had been tested for purity and nodule production. Because of the large number of cultures tested and the desirability of using two media, duplicate determinations were not made. The analyses for total nitrogen were made at the

end of 32 days. Prior to making the final analyses nitrate determinations were made on the uninoculated media and 0.2 mgm. nitrate nitrogen found per 100 c. c. Qualitative tests made on several of the cultures showed no nitrate present, as was to be expected, and hence the reduction method to include nitrates in the total nitrogen determinations was used only in the analysis of the control flasks. The data are given in Table 2.

TABLE 2.—*Results of nitrogen determinations made on various Rhizobium cultures grown on two different media and incubated at 25° C. for 32 days*

Culture	Ashby's medium			Löhnis' soil-extract medium		
	Growth observations	N	N fixed	Growth observations	N	N fixed
		Mgm.	*Mgm.*		*Mgm.*	*Mgm.*
Arlington soybean	Slight	0.02	0.02	Fair	2.00	0.17
Virginia soybean	do	.06	.06	Slight	1.81	−.02
Tokyo soybean	do	.03	.03	Good	1.70	−.13
Kidney bean	do	.02	.02	do	1.83	0
Japan clover	do	.07	.07	Slight	1.81	−.02
Vetch	do	.03	.03	Good	1.85	.02
Crimson clover	do	.06	.06	do	2.14	.31
Trifolium agarium	do	.04	.04	do	1.71	−.12
Velvetbean	do	0	0	Slight	1.76	−.07
Admiral garden pea	do	.02	.03	Good	1.77	−.06
Lima bean	do	.03	.03	Slight	2.11	.28
Alfalfa	do	.01	.01	do	2.14	.31
Petit pois garden pea	do	0	0	do	1.81	−.02
Red clover	do	.01	.01	Good	1.83	0
Sweetclover	do	.01	.01	Slight	1.85	.02
Lotus corniculatus	do	.02	.02	do	1.85	.02
Sulla	do	.01	.01	do	1.70	−.13
Cowpea	do	.01	.01	do	2.07	.24
Garden bean	do	0	0	Fair	1.78	−.05
New Era cowpea	do	−.01	−.01	Slight	1.83	0
Check, uninoculated		0			1.88	
Do		0			1.83	
Do					1.79	

The results seem to show, beyond any reasonable degree of doubt, that under the conditions of the experiment no nitrogen was fixed by the cultures. In the case of Ashby's medium the analyses agree unusually closely and all variations from the control are easily within experimental error. The analyses of the soil-extract medium show slightly wider variations, there being a few increases over the check of 0.2 to 0.3 mgm. N and also a few losses. The average of all the results gives a figure slightly on the positive side, but the increase is not significant. Observations of growth of the various cultures showed no correlation with the nitrogen determinations; for instance, most of those which gave 0.2 to 0.3 mgm. N increase over the control were usually the ones which showed almost negligible growth.

In addition to the results reported here, six other cultures on Mazé's bean-extract medium were run at the same time. The analyses of these showed slight losses in all cases.

NITROGEN-FIXATION STUDIES ON "RADICICOLA" SOLUTION

A medium which has been used frequently for the growing of legume bacteria is "Radicicola" solution, mentioned by Lipman and Brown in their Laboratory Guide for Soil Bacteriology. It consists of 10 gm. of saccharose and 1 gm. of dipotassium phosphate, dissolved in 1 liter

of tap water. In order to expose a large surface of the medium to the air, 100 c. c. portions were placed in 2-liter Erlenmeyer flasks. The flasks were inoculated with six strains of Rhizobia and allowed to incubate for 97 days at room temperature. The period was made long because some of the investigators previously mentioned reported greater fixations over long intervals. Good gum formation was obtained for the two red-clover cultures and with the soybean and cowpea bacteria. The total nitrogen determinations are given in Table 3.

TABLE 3.—*Results of nitrogen determinations on various Rhizobium cultures grown on Radicicola solution and incubated at room temperature for 97 days*

Culture	Growth observations	N	Average N	N fixed
		Mgm.	*Mgm.*	*Mgm.*
Red clover (1)	Fair	0.39	0.44	−0.07
Red clover	do	.49		
Red clover (2)	do	.62	.61	.10
Red clover	do	.59		
Winter pea	Slight	.39	.44	−.07
Do	do	.49		
Sweetclover	do	.60	.53	.02
Do	do	.46		
Soybean	Fair	.39	.49	−.02
Do	do	.58		
Cowpea	do	.56	.56	.05
Do	do	.56		
Control, uninoculated		.46	.51	
Do		.56		

The evidence again shows very definitely that no nitrogen was fixed with any of the cultures, the variations in all cases being within experimental error. This was to a certain extent unexpected prior to analysis because the growth in the flasks seemed unusually good in some cases. The analyses furnish an explanation for this growth, because the medium used was not nitrogen-free as supposed. The sugar used was a commercial product, thus accounting for the presence of the nitrogen.

The data presented in Tables 1, 2, and 3 are typical of a large number of determinations made under very similar conditions. It has been the practice over a period of about five years to include in practically every experiment a check consisting of Ashby's nitrogen-free medium containing various sugars and inoculated with some strain of Rhizobium. In all, about 30 strains have been tested in this way. In no instance has any appreciable growth been observed, other than gum formation, nor have any increases in nitrogen been found that could safely be considered greater than experimental error. Under exactly the same conditions flasks were frequently included which contained the same medium except for the addition of a suitable source of nitrogen, and in most cases at least fairly good growths were secured. Approximately 700 culture flasks containing a wide variety of nitrogen-free media, inoculated with numerous strains of nodule bacteria, and incubated at various temperatures, were discarded without analysis because the many analyses made agreed in showing that there is no need to analyze cultures which show but little growth. Assuming that other conditions are satisfactory, the growth of Rhizobia is roughly propor-

tional to the quantity of available nitrogen present, that is, up to the point where its nitrogen-hunger is largely appeased.

NITROGEN-FIXATION STUDIES WITH SOYBEAN BACTERIA GROWN ON VARIOUS MEDIA

In the studies reported in this paper an attempt was made to include experiments which covered as many variables as possible in the hope that under some given set of conditions the nitrogen-fixing powers of the nodule bacteria might be demonstrated. Naturally the medium received the greatest attention. Some of the data which bear upon this factor are given below.

Five media which had been recommended for the growth of the organism by some research worker and which seemed best adapted for use in fixation experiments were chosen. A description of these, together with the final analyses made after 22 days incubation, is given in Table 4. Unfortunately, the soil-extract media were not analyzed by the modified method to include nitrates. Under such conditions most of the nitrate nitrogen present would appear in the results as "fixation" because the nitrate not determined in the control would appear in the analyses of the bacterial cultures after having been converted into organic forms. Very likely the 0.22 mgm. N reported as fixed in medium 5 was really not fixation at all. The other data of Table 4 show no fixation and all variations are easily within experimental error.

TABLE 4.—*Results of nitrogen determinations on various media inoculated with soybean bacteria media and incubated 22 days*

Medium No.	Composition of medium in grams per liter	Growth observations	N	Average N	N fixed
			Mgm.	*Mgm.*	*Mgm.*
1	Hot water extract of 14 gm. sweetclover plants (tops and roots) and dipotassium phosphate, 0.5 gm., sucrose, 10 gm., adjusted to neutrality.	[1]	[1] 1.48 [1] 1.45	1.47	
		Good	1.56 1.41	1.49	0.02
2	Hot water extract of 50 gm. soil and sucrose 10 gm.	[1]	[1] .46 [1] .44	[2] .45	
		Fair	.44 .44	[2] .44	−.01
3	Lipman's modified Radicicola solution: Sucrose, 10 gm., dipotassium phosphate, 2 gm.; magnesium sulphate, 0.1 gm.; sodium chloride, 0.5 gm.	[1]	[1] .05 [1] .07	.06	
		Slight	.08 .11	.10	.04
4	Lipman's Radicicola solution: Sucrose, 10 gm.; dipotassium phosphate, 1 gm.	[1]		[1] .06	
		Slight	.08 .08	.08	.02
5	Joshi's soil extract; Hot water extract of 250 gm. soil; mannit, 10 gm.; dipotassium phosphate, 0.5 gm.	[1]	[1] 2.76 [1] 2.78	[2] 2.77	
		Fair	3.01 2.96	[2] 2.99	.22

[1] Controls, uninoculated. [2] No allowance made for nitrates in soil.

NITROGEN-FIXATION STUDIES ON VARIOUS CARROT-EXTRACT MEDIA INOCULATED WITH NODULE BACTERIA

It is a well-known fact that many plant extracts, both legume and nonlegume, when present in media greatly increase the growth of Rhizobia. The writer (*1*) found that carrot extract was one of the best for this purpose. It was decided, therefore, to select several

media which have been recommended for the growth of these bacteria and modify them by adding carrot extract to each. This would, of course, give media of fairly high nitrogen content but also media that would, presumably, show very excellent growths. Since previous results had shown no fixation in media low in nitrogen it was desired to determine if fixation would occur in nitrogen-rich media, as many workers have reported. The composition of the media as used, together with the analyses at the end of 25 days, are given in Table 5.

TABLE 5.—*Results of nitrogen determinations on cultures of legume bacteria grown on different media, with the addition of carrot extract, and incubated 25 days*

Medium No.	Composition of medium in grams per liter	Culture	Growth observations	N	N fixed
				Mgm.	*Mgm.*
1	Löhnis (modified): Carrot extract, dipotassium phosphate, 0.5 gm.; mannit, 10 gm.; $CaCO_3$ in excess.	Red clover	Very good	5.60	−0.22
		Kidney bean	Good	5.43	−.39
		Garden bean	do	5.49	−.33
		Trifolium agarium	do	5.49	−.33
2	Moore's synthetic (modified): Carrot extract, sucrose, 10 gm.; dipotassium phosphate, 1 gm.; magnesium sulfate, 0.2 gm.; tap water, reaction not adjusted.	Red clover	No growth	5.80	−.02
		Kidney bean	Fair	5.60	−.22
		Garden bean	Good	5.72	−.10
		Trifolium agarium	do	5.66	−.16
3	Same as medium 2 except adjusted to 4, Fuller's scale.	Red clover	do	5.90	.08
		Kidney bean	do	5.72	−.10
		Garden bean	do	5.72	−.10
		Trifolium agarium	do	5.83	.01
4	Mazé's (modified): Carrot extract, sucrose 20 gm.	Red clover	Fair	5.90	.08
		Kidney bean	Slight	5.62	−.20
		Garden bean	No growth	5.71	−.11
		Trifolium agarium	Fair	5.63	−.19
5	Lipman's Radicicola solution (modified): Carrot extract, sucrose 10 gm., dipotassium phosphate 2 gm., $MgSO_4$, 0.1 gm., NaCl, 0.5 gm.	Red clover	Slight	5.64	−.18
		Kidney bean	Fair	5.76	−.06
		Garden bean	Good	5.76	−.06
		Trifolium agarium	do	5.59	−.23
6	Lipman's Radicicola solution (modified): Carrot extract, dipotassium phosphate, 1 gm., tap water.	Red clover	Fair	5.57	−.25
		Kidney bean	Good	5.55	−.27
		Garden bean	Very good	5.40	−.42
		Trifolium agarium	Good	5.51	−.31
7	Joshi's (modified) medium: Carrot extract, mannit, 10 gm., dipotassium phosphate, 1 gm., tap water.	Red clover	do	5.62	−.26
		Kidney bean	do	5.67	−.15
		Garden bean	Very good	5.50	−.32
		Trifolium agarium	Good	5.76	−.06
8	Ashby's (modified) medium: Carrot extract, dipotassium phosphate, 0.2 gm., NaCl, 0.2, gm., $MgSO_4$, 0.2 gm., $CaSO_4$, 0.1 gm., $CaCO_3$, 1 gm., sucrose, 20 gm.	Red clover	Fair	5.59	−.23
		Kidney bean	Good	5.57	−.25
		Garden bean	Very good	5.44	−.38
		Trifolium agarium	do	5.57	−.25
9	Same as medium 8 except for addition of $MnSO_4$, 0.02 gm., and FeCl$_3$, 0.00025 gm.	Red clover	Fair	5.50	−.32
		Kidney bean	Good	5.50	−.32
		Garden bean	Very good	5.53	−.29
		Trifolium agarium	Good	5.55	−.27
	Control, uninoculated			5.80 5.79 5.85 5.84 — 5.82	

Very little comment on the analyses is necessary. With all four strains of the legume organism good growths were secured on practically all of the media and yet no fixation was obtained. Most of the analyses show slight losses rather than gains. No explanation is offered for this except to state that variations, such as those found, or even greater, are very common in the case of Kjeldahl analyses on plant extracts. The growing of bacterial cultures usually increases the variations.

NITROGEN-FIXATION STUDIES WITH RED-CLOVER BACTERIA GROWN IN HEATED AND UNHEATED CLOVER EXTRACT

The assumption has frequently been made that legume bacteria fix nitrogen by means of enzymes. If the host plant should secrete the enzymes rather than the bacteria, it would be expected that the use of the unheated extract of the host plant in culture media might favor fixation by the bacteria growing independent of the higher plants. This experiment was planned, therefore, to determine if there is any difference in the growth or fixation, if any, of the nodule bacteria growing in the heated and unheated juices of the host plant.

About 400 gm. of the whole red-clover plant, including the roots and nodules, were ground in a meat chopper, water added, and the mass filtered. A sterile Pasteur-Chamberland filter was then used to refilter the solution in order to sterilize it without heating. The filtrate was incubated for a few days and found to be sterile, about 650 c. c. of the sterile diluted plant juice being obtained. Half of this was then autoclaved at 15 pounds pressure for 20 minutes and the two portions used for the experiment reported in Table 6.

TABLE 6.—*Results of nitrogen determinations on cultures of red-clover bacteria grown in heated and unheated clover-extract media*

Medium and treatment	Quantity of extract	Incubated 19 days			Incubated 37 days	
		Growth observations	N	N fixed	N	N fixed
	C. c.		*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*
Control, uninoculated	50		35.81			
	50		36.25			
Without clover extract	0	Slight	.04	0.04	0.08	0.08
	0	....do	.05	.05	.15	.15
Clover extract, unheated	5	Fair	3.68	.08	3.79	.19
	10	....do	7.40	.19	7.26	.05
	20	Good	14.42	.01	14.77	.36
	50	Very good	37.13	1.10	36.75	.72
Clover extract, heated	5	Fair	3.59	−.01	3.74	.14
	10	....do	7.34	.13	7.42	.21
	20	Good	14.59	.18	14.70	.29
	50	Very good	36.65	.62	37.12	1.09

The data show no difference in growth between the heated and unheated media, both producing excellent viscous growths, roughly agreeing with the quantity of plant extract added. The analyses show no gains in nitrogen worthy of note. It is true that with the largest quantity of plant extract added the analyses show an average of about 0.9 mgm. of nitrogen increase, but considering that in this case a large quantity of nitrogen was present, the 0.9 mgm. is scarcely significant. Since these results were obtained the writer has had occasion to make many analyses of plant extracts and is thoroughly convinced that a variation of 1 mgm. nitrogen is not significant where as much as 35 mgm. N is present. Duplicate analyses commonly agree more closely than this for uninoculated material, but not after a considerable portion of the nitrogen in the medium has been converted into miscellaneous forms as a result of bacterial growth. It seems safe to conclude that probably no nitrogen fixation took place in this experiment, particularly in view of the fact that it was only in those flasks which contained perhaps five times as much combined nitrogen as the bacteria needed that the small increases were secured.

NITROGEN-FIXATION STUDIES WITH RED-CLOVER ORGANISM GROWING ON A SAND SLOPE

An experiment was next started to test the rather generally accepted idea that Rhizobia fix more nitrogen if the small quantity fixed is constantly removed, as is commonly believed to occur in the nodule. In order to do this a glass tube about 1.5 inches in diameter and 15 inches long was fitted with rubber stoppers, the lower containing one hole and the upper two holes, through which glass tubes passed. This was filled about one-third full of quartz sand, the tube being placed in a slightly elevated position with the sand extending from end to end. After sterilization, a culture of red-clover bacteria was introduced and the apparatus kept at room temperature. Through one of the two tubes at the upper end was constantly sucked a very slow stream of air which passed over the sand rather than through it. To the other tube was attached connections for delivering Ashby's nutrient solution, containing 1 per cent mannit. Twice daily during the course of the experiment 25 to 50 c. c. of the nutrient solution was allowed to run on to the upper part of the sand layer. Three liters of this solution were used, analyses for total nitrogen being made on each 750 c. c. portion as it came through, and finally of the sand. The analyses showed gains of 0.02, 0.07, 0.14, and 0.27 mgm. N, respectively, for the four portions, or a total of 0.5 mgm. The analyses of the sand showed no nitrogen. Practically no growth, outside of a trace of gum formation, was observed during the 25 days duration of the experiment. This was to be expected for a nitrogen-free medium in case no nitrogen was fixed.

The experiment was repeated, using Ashby's medium containing 1 per cent sucrose instead of dextrose. The analyses made at three intervals showed −0.10, 0.37, and 0.20 mgm. N fixed and no nitrogen in the sand. Considering the bulk of solution used and the general procedure followed, the results would seem to be within experimental error and to indicate further that the organism probably does not fix much, if any, nitrogen independent of the host.

NITROGEN-FIXATION STUDIES IN CARROT EXTRACT BY STRAINS OF RHIZOBIA

Since previous experiments had shown that the legume bacteria grow well only when in a medium containing fixed nitrogen, particularly certain plant extracts, it seemed desirable to run additional experiments using such media. For these experiments, therefore, a carrot-extract medium was selected. This was prepared by boiling the roots, after passing them through a meat chopper, for a half hour in 2 parts of water to 1 of carrots. The extract was filtered and the clear filtrate added to 100 c. c. portions of Ashby's medium, at the rates of 5 and 25 c. c. The Ashby's solution in this case contained 2 per cent sucrose. After sterilization, different culture flasks were inoculated with 20 cultures of nodule bacteria. In order to insure that conditions were optimum for growth and fixation, two flasks were also inoculated with *Azotobacter vinelandii*. Because of the large number of cultures used, duplicate inoculations were not made. After incubation for 14 days, analyses for total nitrogen were made, these being given in Table 7.

TABLE 7.—*Results of nitrogen determinations on cultures of Rhizobium grown on Ashby's medium containing 2 per cent sucrose and varying quantities of carrot extract and incubated for 14 days*

Culture	5 c. c. carrot extract added per 100 c. c.			25 c. c. carrot extract added per 100 c. c.		
	Growth observations	N	N fixed	Growth observations	N	N fixed
		Mgm.	*Mgm.*		*Mgm.*	*Mgm.*
Kidney bean	Fair	2.12	−0.08	Very good	10.94	−0.06
Garden pea	do	2.27	.07	do	11.08	.08
Lima bean	do	2.17	−.03	Good	10.69	−.31
Garden pea	Slight	2.06	−.14	do	10.47	−.53
Cowpea	Fair	2.30	.10	Fair	10.74	−.26
Crimson clover	do	2.40	.20	Very good	11.24	.24
Soybean	do	2.32	.12	Fair	10.69	−.31
Cowpea	do	2.19	−.01	do	10.78	−.22
Red clover	Good	2.15	−.05	Very good	10.89	−.11
Sweetclover	Fair	2.14	−.06	do	11.27	.27
Vetch	do	2.12	−.08	do	10.36	−.64
Trefoil	Good	2.15	−.05	do	11.03	.03
Sulla	Fair	2.11	−.09	do	10.47	−.53
Japan clover	Good	2.14	−.06	Good	10.78	−.22
Alfalfa	Fair	2.20	.0	do	10.75	−.25
Soybean	do	2.15	−.05	do	10.43	−.57
Do	do	2.13	−.07	Very good	10.40	−.60
Velvetbean	do	2.23	.03	Good	10.96	−.04
Garden bean	do	2.20	.0	Very good	10.98	−.02
Yellow clover	Good	2.25	.05	do	11.53	.53
Azotobacter vinelandii	do	8.42	6.22	do	19.32	8.32
Control uninoculated		2.33			10.96	
Do		2.07			11.03	

It will be observed that regardless of the fact that nearly all of the cultures of the legume organisms made good growths on the medium used, there is little or no indication that any nitrogen fixation took place. Decreases were more common than increases. These decreases probably do not represent actual losses of nitrogen but merely that the bacterial growths so changed the form of nitrogen present that the Kjeldahl method failed to get all of it. This is common when dealing with plant extracts, and again merely emphasizes the fact that any gains obtained on plant extract or similar media must be relatively large if the data are to be given serious consideration so far as proving that the bacteria can use atmospheric nitrogen.

The nitrogen-fixation rate, as shown in Table 7, was about as expected in the case of *Azotobacter vinelandii*. The fixation would probably have been slightly larger had it not been for the fact that the carrot extract contained considerable nitrogen. The fact that the fixation was 2.1 mgm. N higher where the larger quantity of extract was present shows that the added nitrogen did not greatly interfere with the fixation process. It indicates, further, that the Azotobacter used the carbohydrates present in the extract for growth and fixation.

An experiment, similar to the one reported in Table 7, was next carried out using Ashby's medium containing various quantities of carrot extract and sucrose. A culture of nodule bacteria from yellow clover was used for inoculation; also one of *Azotobacter vinelandii* for comparison. The analyses, made after 23 days, are given in Table 8.

TABLE 8.—*Results of nitrogen determinations on Rhizobium and Azotobacter vinelandii cultures grown on Ashby's medium containing varying quantities of sucrose and carrot extract and incubated 23 days*

Culture	Additions to Ashby's mineral medium per 100 c. c.		Growth observations	N	Average N	N fixed
	Carrot extract	Sucrose				
	C. c.	*Gm.*		*Mgm.*	*Mgm.*	*Mgm.*
	10	0	Good	4.27	4.25	−0.08
	10	0	do	4.23		
	25	0	Very good	10.62	10.63	−.03
	25	0	do	10.63		
	10	.5	Good	4.31	4.28	−.05
	10	.5	do	4.25		
	25	.5	Very good	10.67	10.63	−.03
Yellow clover	25	.5	do	10.58		
	10	1.0	Good	4.24	4.24	−.09
	10	1.0	do	4.23		
	25	1.0	Very good	10.33	10.48	−.18
	25	1.0	do	10.62		
	10	2.0	Good	4.06	4.20	−.13
	10	2.0	do	4.34		
	25	2.0	Very good	10.32	10.50	−.16
	25	2.0	do	10.67		
	10	0	Fair	6.29	6.03	1.70
	10	0	do	5.76		
A. vinelandii	25	0	Good	12.78	14.04	3.38
	25	0	do	15.29		
	25	2.0	Very good	18.69	19.35	8.69
	25	2.0	do	20.00		
	10	0		4.35	4.33	
Control, uninoculated	10	0		4.30		
	25	0		10.70	10.66	
	25	0		10.62		

The duplicate analyses check very closely, considering that a considerable quantity of plant-extract nitrogen was present, and again show no fixation of free nitrogen regardless of the fact that the growth was exceptionally good. Under the same conditions *Azotobacter vinelandii* fixed nitrogen at a normal rate.

Considerably more data similar to that discussed could be given if space permitted. All of these figures bring out the fact that it is impossible to secure very closely agreeing analyses of bacterial cultures growing on plant-extract media. A summation of the negative and positive results gives a figure near zero but with a slight difference on the negative side. All of the evidence shows that the legume organism under these conditions does not fix nitrogen but merely uses that present in a combined form in the plant juice for its growth. In fact, the main explanation for the increased growth of nodule bacteria on plant-extract media is the presence of the nitrogen, probably in a particularly suitable form.

NITROGEN-FIXATION STUDIES WITH MEDIA CONTAINING VARIOUS COMBINATIONS OF INGREDIENTS

Numerous experiments conducted during the course of these investigations showed that the presence of carrot extract, soil extract, or asparagine in media was favorable to the growth of the nodule bacteria. In some cases manganese salts seemed to be stimulating. In order to find out if increases in nitrogen occurred in media con-

taining these materials, it was decided to test out various combinations of these constituents added to media in quantities that previous experiments had shown would be favorable. Just prior to starting this experiment a sample of "bios" was obtained from W. H. Eddy and included in the study. The reason for including the bios was the fact that it is known to be an excellent growth promoter for yeast, and, furthermore, chemical studies with plant extracts had indicated that the stimulating properties of these for Rhizobia might be due partly to their bios content. The various materials were added to Ashby's solution, containing 1 per cent dextrose, sterilized, and inoculated with red-clover bacteria. The analyses for total nitrogen made at the end of 10 days are given in Table 9.

TABLE 9.—*Results of nitrogen determinations of Rhizobia cultures grown in Ashby's media containing bios and other additions and incubated for 10 days*

Additions made to Ashby's minerals in quantities per 100 c. c. media					Growth observations	N	N fixed
Bios	$MnCl_2$	Carrot	Soil	Asparagine			
Mgm.	*Mgm.*	*C. c.*	*C. c.*	*Mgm.*		*Mgm.*	*Mgm.*
0	0	0	0	0	Slight	0	0
0	10	0	0	0	....do	0	0
0	0	10	0	0	Fair	3.08	−.09
0	0	0	10	0	....do	.22	−.02
.25	0	0	0	0	Slight	0	0
0	0	0	0	23	Fair	4.37	−.08
0	10	10	0	0	....do	3.20	.03
0	10	0	10	0	....do	.24	0
.25	10	0	0	0	Slight	.64	.04
0	10	0	0	23	Fair	4.48	.03
0	0	10	10	0	Good	3.38	−.03
0	0	10	0	23	....do	7.44	−.18
.25	0	0	10	0	Fair	.25	.01
0	0	0	10	23	....do	4.47	−.22
0	10	10	10	0	Good	3.56	.15
0	10	10	0	23	....do	7.49	−.13
.25	10	0	10	0	Fair	.25	.01
0	10	0	10	23	....do	4.53	−.16
.25	10	0	0	23	....do	4.32	−.13
0	0	10	10	23	Good	7.66	−.20
.25	0	0	10	23	Fair	4.47	−.22
0	10	10	10	23	Good	7.65	−.21
.25	10	10	10	23	....do	7.61	−.25
[1] 0	0	0	0	23		4.45	
0	0	0	0	23		4.44	
0	0	10	0	0		3.19	
0	0	10	0	0		3.15	
0	0	0	10	0		.24	
0	0	0	10	0		.24	

[1] The last 6 cultures were uninoculated.

It will be observed that the growth varied directly with the quantity of nitrogen present in the medium, there being practically no growth in a nitrogen-free medium, as is always the case with legume bacteria. No fixation occurred in any of the media, all of the results being within experimental error. Aside from nitrogen fixation the results show, further, that Eddy's bios was without appreciable effect, whether tested in the presence or absence of available nitrogen. Practically the same can be said for manganese chloride under the conditions used. Soil extract, asparagine, and carrot extract all increased the growth, due chiefly to their nitrogen content.

NITROGEN-FIXATION STUDIES WITH RED-CLOVER BACTERIA GROWN IN SPINACH EXTRACT

Among the plant extracts which previous work (*1*) had shown to be especially good in promoting growths of Rhizobia was spinach. An experiment was therefore started to determine if this increased growth meant a fixation of atmospheric nitrogen. Both hot and cold water extracts were prepared, the hot-water extract being made by passing 215 gms. of green spinach through a meat chopper, adding water, boiling one hour, filtering, and making up to 1,300 c. c. The cold-water extract was prepared in the same manner except for the absence of heat. These extracts were added in various proportions to Ashby's medium with and without calcium carbonate and sucrose. The sterilized media in 300 c. c. culture flasks were inoculated with a pure culture of red-clover bacteria and allowed to incubate for 18 days at 28° C. before analysis. The results are given in Table 10.

TABLE 10.—*Results of nitrogen determinations on cultures of red-clover bacteria grown on Ashby's medium containing spinach extract, and other additions and incubated 18 days at 28° C.*

Additions to Ashby's medium in quantities per 100 c. c. media			Growth observations	N	N fixed
Spinach extract	$CaCO_3$	Sucrose			
	Gm.	*Gm.*		*Mgm.*	*Mgm.*
0	0.1	1.0	Slight	0.09	0.09
5 c. c. cold	.1	1.0	Fair	1.48	.08
10 c. c. cold	.1	1.0	do	2.74	−.06
25 c. c. cold	.1	1.0	Good	6.72	−.29
50 c. c. cold	.1	1.0	Very good	13.26	−.76
75 c. c. cold	.1	1.0	Doubtful	20.91	−1.99
100 c. c. cold	.1	1.0	do	25.51	−2.41
5 c. c. hot	.1	1.0	Fair	1.17	.14
10 c. c. hot	.1	1.0	do	2.18	.17
25 c. c. hot	.1	1.0	Good	5.50	.57
50 c. c. hot	.1	1.0	Very good	9.63	−.17
75 c. c. hot	.1	1.0	do	14.59	−.08
100 c. c. hot	.1	1.0	Good	18.02	−1.58
25 c. c. cold	.1	0	Doubtful	5.90	−1.11
25 c. c. cold	.1	0	do	5.86	−1.15
5 c. c. hot	0	1.0	Fair	1.14	.11
10 c. c. hot	0	1.0	do	2.16	.15
25 c. c. hot	0	1.0	Good	5.28	.35
50 c. c. hot	0	1.0	Very good	10.03	.23
75 c. c. hot	0	1.0	do	15.13	.46
100 c. c. hot	0	1.0	do	19.83	.29
25 c. c. hot	0	0	Fair	4.53	−.37
0[1]	.1	1.0		0	
50 c. c. hot	0	0		9.87	
50 c. c. hot	0	0		9.73	
50 c. c. cold	0	0		14.02	
50 c. c. cold	0	0		14.02	

[1] The last 5 cultures were uninoculated.

These data furnish little evidence that the nodule bacteria can use free nitrogen. A few slight gains in nitrogen were obtained but the losses were considerably greater. Evidently the bacteria obtained all of the nitrogen needed from the plant extract.

COMPARISON OF MEDIA FOR THE GROWTH OF VARIOUS STRAINS OF RHIZOBIA

An experiment to compare nitrogen fixation, if any, by Rhizobia grown in solution and on agar, using three media, was started. The solution cultures were grown in 100 c. c. portions of media in 500 c. c. Erlenmeyer flasks for a period of 39 days. The agar cultures were

made in Petri dishes, but were not analyzed because of the numerous fungus contaminations which occurred. The solution cultures were analyzed for total nitrogen, however, and these data are given in Table 11.

TABLE 11.—*Results of nitrogen determinations on Rhizobia cultures grown on various solution cultures and incubated for 39 days*

Medium	Culture	Growth observations	N	Average N	N fixed
			Mgm.	*Mgm.*	*Mgm.*
Greig-Smith's with $CaCO_3$: Dextrose 2 per cent Na_2HPO_4, 0.2 per cent $CaCO_3$ 0.5 per cent.	Red clover	Slight	0.34	0.46	0.15
	do	do	.58		
	Soybean	do	.37	.38	.07
	do	do	.38		
	Alfalfa	do	.40	.40	.09
	do	do	Lost.		
	Sulla	do	.36	.36	.05
	do	do	Lost.		
Ashby's with 2 per cent dextrose.	Red clover	Fair	.33	.35	.01
	do	do	.36		
	Soybean	do	.41	.44	.10
	do	do	.47		
	Alfalfa	do	.39	.39	.05
	do	do	.38		
	Sulla	do	.36	.35	.01
	do	do	.34		
Ashby's with 2 per cent dextrose, plus spinach extract.	Red clover	Good	12.95	13.09	.27
	do	do	13.23		
	Soybean	Slight	Lost.		
	do	Contaminated			
	Alfalfa	Fair	12.52	12.50	−.32
	do	do	12.48		
	Sulla	Good	13.12	12.80	−.02
	do	do	12.48		
Control, uninoculated; Greig-Smith's.			.28	.31	
			.34		
Control, uninoculated; Ashby's			.34	.34	
			.34		
Control, uninoculated; Ashby's with spinach.			13.01		
			12.77		
			12.77	12.82	
			12.74		

The results merely substantiate data previously given and show no fixation by any of the four cultures of Rhizobia grown on the three media, the variations being within experimental error. Duplicate determinations failed to agree quite as closely in this experiment as in most of the others given in this paper, but the variations between duplicates were in only one case greater than 0.28 mgm. nitrogen. Unfortunately four of the determinations were lost due either to breakage of flasks or contamination of cultures.

USE OF VARIOUS CARBOHYDRATES BY RHIZOBIA

Most of the experimental work with legume-nodule bacteria, reported above, was conducted in nutrient media containing dextrose as the source of energy. In some cases miscellaneous carbohydrates, found in various plant juices, have also been present in varying percentages. In other experiments, not reported here, the growth of the organism on other sugars, including the hydrolyzed products of starches, gums, cellulose, and miscellaneous materials, has been determined. Practically all of these mixtures served as suitable energy sources but seemed to be no better than any one of the four sugars commonly used, namely, sucrose, dextrose, mannit, and maltose. As an additional check on this, and also in order to determine if nitrogen fixation occurs with any of the carbohydrates, the experiment reported in Table 12 was conducted. Four media were used: (1) Ashby's minerals with the various energy sources, (2) Ashby's

with 0.05 per cent potassium nitrate for the purpose of serving as a check on growth, (3) Ashby's with 0.2 per cent egg albumen, and (4) Ashby's with 0.2 per cent egg albumen and 0.05 per cent potassium nitrate. The reason for the use of the albumen was that in rather extensive studies, not yet reported, it was found that a certain commercial egg albumen served to increase the growth of certain strains of Rhizobia, provided an available form of nitrogen was also present in the medium. In the absence of the fixed nitrogen the albumen had very little effect. It seemed, therefore, that this would be an ideal method of testing the organism for nitrogen-fixing powers and far superior to the use of a plant extract of variable composition. In Ashby's solution containing certain brands of commercial egg albumen and nitrogenous salts the conditions for the growth of some strains of Rhizobia seem to be nearly ideal and in many cases superior to plant-extract media. It was further determined that the egg albumen was not used to any appreciable extent, if at all, by the bacteria. Apparently either the impurities present or the physical properties of the albumen were responsible for the beneficial effects on bacterial growth. By omitting all available forms of nitrogen it would seem that conditions would be ideal for fixation if the organism possesses this ability. As a check on the culture work a series of flasks was also included in which both egg albumen and potassium nitrate were present. The cultural methods used were the usual ones, the culture being a red-clover organism which was grown in 100 c. c. of the various media containing 1 per cent of the carbohydrate. After incubation at 28° C. for a period of 65 days the egg albumen series was analyzed for total nitrogen. Analyses were not made on the series containing Ashby's nitrogen-free medium because the growth was almost negligible in all cases. Neither were analyses made on the other two series, containing nitrogen, carried along largely for comparison. The observations and data are given in Table 12.

TABLE 12.—*Results of nitrogen determinations on cultures of the red-clover strain of Rhizobia grown on different media in the presence of various carbohydrates*

Carbohydrate	Ashby's nitrogen-free medium, growth observations	Ashby's medium with 0.05 per cent KNO_3, growth observations	Ashby's medium with 0.2 per cent egg albumen			Ashby's medium with 0.2 per cent egg albumen and 0.05 per cent KNO_3, growth observations
			Growth observations	N	N fixed	
				Mgm.	*Mgm.*	
Dextrose, commercial	Slight	Good	Fair	25.00	0.19	Very good.
Sucrose, commercial	do	do	do	25.09	.28	Do.
d-Mannitol, c. p.	do	do	do	24.96	.15	Do.
Lactose, c. p.	do	Fair	do	24.65	−.16	Do.
d-mannose, c. p.	do	do	do	24.96	.15	Do.
l-arabinose, c. p.	do	Slight	do	24.73	−.08	Good.
l-xylose, c. p.	do	do	do	24.52	−.29	Do.
d-galactose, c. p.	do	Fair	do	24.70	−.11	Do.
Raffinose, c. p.	do	do	Slight	24.96	.15	Do.
Maltose, c. p.	do	Contaminated	Fair	24.96	.15	Very good.
d-glucose, c. p.	do	Fair	do	25.09	.28	Do.
Glycerine, c. p.	do	do	do	24.79	−0.2	Do.
Dextrin, commercial	do	do	do	Lost.		Do.
Soluble starch (Lintner).	No growth	No growth	No growth			No growth.
Starch, commercial	do	do	do			Do.
Gum arabic	do	do	do			Do.
Control for analysis				24.89		
Do				24.59		
Do				24.95		

The results of the analyses were all within experimental error, with the slight apparent gains in a few instances approximately balancing the slight losses obtained in others. With conditions ideal for growth, except for the absence of a supply of available nitrogen, no fixation occurred. All of the carbohydrates used, with the exception of starch and gum arabic, apparently served as suitable sources of energy. This statement is, however, based on observations only, since no sugar determinations were made.

NITROGEN-FIXATION STUDIES WITH LEGUME-NODULE BACTERIA GROWING ON AGAR

In some of the investigations which have been reported in the literature showing nitrogen fixation by Rhizobium, the cultures were grown on agar media. In general, the observations indicated that the growth was better under such conditions than where the organisms were in solution cultures, and the fixation in some instances was reported as greater. These findings would seem to be logical in case the organism fixes nitrogen, because on the host plant the organisms are not submerged in water. The root nodules are normally exposed to the soil air; in fact, when submerged for any great length of time they usually cease to function, or at least fixation proceeds at a slower rate. The writer has frequently observed that the organisms on agar cultures often seem to grow much better in the laboratory than do those in solution cultures, but this may be merely because the organisms and the gum are more apparent on agar. Certainly the organisms die out sooner in solution.

A preliminary experiment was started, which need not be reported in detail here, using three media and four cultures of Rhizobia. These cultures were made in Petri dishes and also in some large flat-bottomed culture dishes, similar in shape to Petri dishes. A large number of these became contaminated with fungi before the end of the incubation period. The uncontaminated cultures, practically all of which showed excellent growth with good gum formation, were analyzed for total nitrogen and the results were so near those for the uninoculated control as to be within the limits of experimental error.

Another experiment was then started, using Ashby's medium to which was added 15 gm. of dextrose and 10 gm. of agar per liter. Three-liter Erlenmeyer flasks were used as culture flasks, each containing 100 c. c. of the medium. A culture of alfalfa bacteria was used for inoculation. After incubation for 48 days at room temperature, total nitrogen determinations were made. These are given in Table 13.

TABLE 13.—*Results of nitrogen determinations on cultures of alfalfa bacteria grown on a solid agar medium and incubated at room temperature for 48 days*

Culture	Growth observations	N	Average N	N fixed
		Mgm.	*Mgm.*	*Mgm.*
Control, uninoculated		2.73 2.84 2.73 2.69	2.75	
Alfalfa bacteria	Good	2.82 2.84 2.96	2.87	0.12

The average gain in nitrogen was in this case 0.12 mgm. N, which is not enough to be significant. It seems that the organism was able to secure enough nitrogen from the agar to make a rather good growth. The actual mass of bacterial cells produced was probably much smaller than might be expected from a casual glance at the gummy cultures.

A repetition of the experiment, reported above, was then started, using five cultures of legume bacteria. The medium used was Ashby's with and without calcium carbonate at the rate of 2 gm., dextrose 20 gm., and agar 10 gm. per liter. Three-liter flasks, containing 100 c. c. of the above media, were used as culture flasks. These were kept at room temperature for 33 days and analyses then made for total nitrogen. These figures are given in Table 14.

TABLE 14.—*Results of nitrogen determinations on cultures of several strains of nodule bacteria grown on an agar medium and incubated at room temperature for 33 days*

Culture	Calcium carbonate added	Growth observations	N	Average N	N fixed
	Grams per 100 c. c.		*Mgm.*	*Mgm.*	*Mgm.*
Control, uninoculated	0.2		3.03	3.02	
	0		2.98		
	0		3.04		
Red clover	.2	Good	2.86	2.95	−0.07
	.2	do	3.04		
	0	Fair	2.94	2.94	−.08
Sweetclover	.2	Good	2.86	2.90	−.12
	.2	do	2.93		
	0	Fair	2.93	2.93	−.09
Soybean	.2	do	2.91	2.98	−.04
	.2	do	3.04		
	0	do	2.94	2.94	−.08
Alfalfa	.2	Good	2.93	2.99	−.03
	.2	do	3.04		
	0	do	2.98	2.98	−.04
Winter pea	.2	Fair	2.93	2.93	−.10
	0	do	3.00	3.00	−.03

The conclusions to be drawn from these results are essentially the same as from Table 13. The analyses checked exceptionally closely, the variations between the extremes being only 0.18 mgm. N. Certainly these results are within experimental error and again demonstrate that under the experimental conditions no free nitrogen was fixed by any of the five strains of bacteria. Good growths, considering the medium used, were obtained with three of the cultures and fair growths with the other two. The agar used was a well-purified sample, but presumably the bacteria were able to obtain some available nitrogenous material from it.

As a further test of the nitrogen-fixing power of nodule bacteria another experiment was conducted, using Ashby's mineral solution with the addition of 1 per cent mannit and 1 per cent agar. This medium was added to 3-liter Erlenmeyer flasks at the rate of 200 c. c. each and sterilized. Ten pure cultures of nodule bacteria were used for inoculation; also a culture of *Azotobacter vinelandii* for comparison. The cultures were incubated at 28° C. for 32 days, after which analyses were made by the Kjeldahl method, using mercury as a catalyst in digestion and sodium thiosulphate for the precipitation of the mercury prior to distillation. Table 15 gives the growth observations and final analyses.

TABLE 15.—*Results of nitrogen determinations on cultures of Rhizobia, and of Azotobacter vinelandii, grown on thin agar layers and incubated at 28° C. for 32 days*

Culture	Growth observations	N	Average N	N fixed
		Mgm.	*Mgm.*	*Mgm.*
Soybean	Fair, very gummy	3.67 3.56 3.64	3.62	0.09
Clover	Good, very gummy	3.49 3.42 3.56	3.49	−.04
Pea	Fair	3.51 3.40 3.50	3.47	−.06
Kidney bean	do	3.58 3.58 3.37	3.51	−.02
Alfalfa	Good	3.51 3.44	3.48	−.05
Lespedesa	Slight	3.51 3.44	3.48	−.05
Velvetbean	Fair	3.50 3.63	3.57	.04
Dalea	Good	3.62 3.54	3.58	.05
Sulla	do	3.40 3.67	3.54	.01
Sweetclover	Fair	3.39 3.59	3.49	−.04
A. vinelandii	Very good	21.20 17.36	19.28	15.75
Control, uninoculated		3.65 3.51 3.54 3.50 3.46	3.53	

The organisms made good growths in most cases, considering that they were grown under nitrogen-starvation conditions. The term "good" as used in the table may be misleading, however. In no case was a growth obtained comparable to those ordinarily secured in a medium containing adequate nitrogen. In this case good simply means that the organisms produced a glistening layer of gum on the surface of the agar. Actually the organisms were not making an abundant growth such as would be produced on a suitable plant extract or similar medium.

The evidence in Table 15 shows very conclusively that no nitrogen was fixed by any of the 10 strains of Rhizobia. All results were within experimental error. On the other hand, *Azotobacter vinelandii* fixed an average of 7.87 mgm. N per gram of mannit. This is exceptionally high and is the largest fixation per unit of sugar ever secured by the writer with an Azotobacter culture. The fact that the medium and growing conditions were so exceptionally favorable for nitrogen fixation makes the negative results with Rhizobia seem all the more significant.

NITROGEN-FIXATION STUDIES ON SOIL-EXTRACT MEDIA

A review of the work that has been done with legume-nodule bacteria brings out the fact that these organisms respond in many cases to low concentrations of certain mineral elements not ordinarily added to synthetic nutrient media. Several experiments were made to test out this point with various concentrations of elements, such as Cu, Mn, B, Zn, Pb, etc. There was little to indicate, however, that such elements play an important rôle in nitrogen fixation by nodule bacteria growing in culture flasks. It is true, though, that soil-extract media, which contain traces of many mineral salts, have been reported as favorable to growth and to fixation by the legume

bacteria. Such extracts contain appreciable quantities of nitrogenous salts, which might explain the increased growth effects, but there still seems to be evidence that the mineral constituents present also increase nitrogen fixation by Rhizobia. In fact, soil-extract media have been more or less generally recognized as the best to use in demonstrating the nitrogen-fixing power of these bacteria. Several experiments were, therefore, conducted to determine if the nodule bacteria can fix nitrogen when growing in such media. In all of the experiments which follow the soil extract was prepared by autoclaving the soil for one hour in the presence of a few grams of calcium carbonate and 2 parts of water to 1 of soil by weight. Enough additional water was used to leach out the soil on the filter to make the final weight of soil extract twice that of the original soil.

The medium used in the first experiment consisted of soil extract added in various proportions to a mineral-sugar medium containing 1 per cent mannit, 0.05 per cent K_2HPO_4, and 0.1 per cent $CaCO_3$. The experiment was carried out in 500 c. c. Erlenmeyer flasks, each containing 200 c. c. of the soil-extract medium. Six strains of Rhizobia were used for inoculation, the analyses for total nitrogen being made after an incubation period of 21 days. Tests for nitrate nitrogen in the soil extract were made, but the quantity present was too small to affect the Kjeldahl analysis, hence the ordinary method of analysis using K_2SO_4 and $CuSO_4$ was used. The results of these nitrogen determinations, together with the observations of growth, are given in Table 16. Duplicate determinations were not made in this case because of the large number of variables introduced.

TABLE 16.—*Results of nitrogen determinations on cultures of nodule bacteria grown on a mineral-sugar medium containing soil extract and incubated 21 days*

Culture	Growth observations	Nitrogen content of sterile media	Nitrogen content of inoculated media	Nitrogen fixed
		Mgm.	*Mgm.*	*Mgm.*
Red clover	Slight	0	0	0
	Fair	.83	.88	.05
	do	1.66	1.61	−.05
	Good	3.32	3.14	−.18
	Very good	8.30	8.56	.26
	do	16.60	16.52	−.08
Crimson clover	Slight	0	0	0
	do	.83	.86	.03
	Fair	1.66	1.61	−.05
	do	3.33	3.52	.19
	Good	8.30	8.65	.35
	do	16.60	16.72	.12
Alfalfa	Slight	0	0	0
	Fair	.83	.91	.08
	do	1.66	1.54	−.12
	Good	3.33	3.28	−.05
	Very good	8.30	8.34	.04
	do	16.60	16.25	−.35
Soybean	Slight	0	.03	.03
	do	.83	.98	.15
	Fair	1.66	1.59	−.07
	do	3.33	3.44	.11
	do	8.30	7.82	−.48
	do	16.60	15.90	−.70
Cowpea	Slight	0	.07	.07
	do	.83	.97	.14
	Fair	1.66	1.64	−.02
	do	3.33	3.48	.15
	do	8.30	8.03	−.27
	do	16.60	16.40	−.20
Vetch	Slight	0	.08	.08
	Fair	.83	.89	.06
	do	1.66	1.35	−.31
	Good	3.33	Lost.	
	Very good	8.30	7.96	−.34
	do	16.60	16.17	−.43

The results show that in most cases the growth of the organisms was roughly proportional to the nitrogen content of the medium in which they grew. This was particularly true for the red clover, alfalfa, and vetch strains. The cowpea and soybean cultures made poor growths, as is always the case with these strains. The analyses show no evidence to indicate that any of the strains could fix free nitrogen. A few slight increases in nitrogen were obtained, but in more cases losses were secured. The largest variations were usually where there was more nitrogen present than the bacteria needed. Evidently the stimulating effect of soil extract for nodule bacteria is primarily due to the nitrogen added.

TABLE 17.—*Results of nitrogen determinations on cultures of Rhizobia grown on a modified Ashby's mineral solution containing various amounts of soil extract*

Culture	Quantity of soil extract per 100 c. c.	Incubated 18 days				Incubated 31 days				Average N fixed
		Growth observations	N	Average N	N fixed	Growth observations	N	Average N	N fixed	
	C. c.		*Mgm.*	*Mgm.*	*Mgm.*		*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*
Red clover	0	Slight	0.06	−0.03	−0.03	Slight	−0.02	−0.03	−0.03	−0.03
	0	do	−.13			do	−.04			
	5	do	−.01	−.05	−.14	do	.08	.10	.01	−.07
	5	do	−.10			do	.13			
	10	Fair	−.02	.08	−.10	Fair	.14	.15	−.03	−.07
	10	do	.19			do	.17			
	25	do	.56	.54	.08	do	.48	.50	.04	.06
	25	do	.53			do	.53			
	75	Good	1.52	1.44	.07	Good	1.45	1.45	.08	.08
	75	do	1.36			do	Lost.			
Cowpea	0	Slight	.12	.06	.06	Slight	.07	.03	.03	.05
	0	do	0			do	−.02			
	5	do	.11	.12	.03	do	.02	−.04	−.13	−.05
	5	do	.13			do	−.09			
	10	do	0	.04	−.14	do	.20	.20	.02	−.06
	10	do	.08			do	Lost.			
	25	Fair	.40	.48	.02	Fair	.47	.47	.01	.02
	25	do	.57			do	.46			
	75	do	1.52	1.52	.15	do	1.46	1.44	.07	.11
	75	do	1.52			do	1.41			
Soybean	0	Slight	.02	.01	.01	Slight	−.01	−.03	−.03	−.01
	0	do	0			do	−.05			
	5	do	.07	.13	.04	do	.02	.07	−.02	.01
	5	do	.19			do	.11			
	10	Fair	.35	.35	.17	Fair	.17	.17	−.01	.08
	10	do	Lost.			do	.17			
	25	do	.53	.47	.01	do	.47	.46	0	.01
	25	do	.40			do	.44			
	75	Good	1.27	1.39	.02	Good	1.39	1.43	.06	.04
	75	do	1.50			do	1.47			
Alfalfa	0	Slight	0	.02	.02	Slight	−.05	−.07	−.07	−.03
	0	do	.04			do	−.08			
	5	do	.04	.02	−.07	do	.04	.05	−.04	−.06
	5	do	0			do	.06			
	10	do	.07	.04	−.16	do	Lost.	.08	−.10	−.13
	10	do	0			do	.08			
	25	Fair	.21	.17	−.29	Fair	.40	.39	−.07	−.18
	25	do	.13			do	.38			
	75	Good	1.56	1.36	−.01	Good	1.38	1.40	.03	.01
	75	do	1.15			do	1.41			
Uninoculated	50						.91			
	50						.89			
	50						.94	.91		

A second experiment dealing with nitrogen-fixing powers of various strains of Rhizobium was made in Ashby's mineral solution containing 2 per cent dextrose, 0.05 per cent K_2HPO_4 instead of 0.02 per cent, and various quantities of soil extract. Two-liter Erlenmeyer flasks containing 100 c. c. of the medium were used for the culture work. The soil extract was prepared in the usual manner, except that the soil was first leached with cold water to remove the nitrates. This was done in order to avoid the necessity of using the much longer and more difficult reduction method for including nitrate nitrogen. While this method is accurate, the experimental error is usually larger, due to the longer procedure used. This is especially true where solutions are analyzed which contain materials, such as sugars or calcium carbonate, which cause frothing. Several comparisons of the Kjeldahl method, in which potassium sulphate, copper sulphate, and sulphuric acid were used in the digestion flask with the older method in which metallic mercury and sulphuric acid were used, seemed to give slightly better results on soil-extract media with the latter method. This method of analysis was, therefore, used in this experiment. Sufficient sodium thiosulphate was added prior to distillation to precipitate the mercury. Analyses were made at the end of both 18 and 31 days, the results together with the observations of growth being given in Table 17.

The growth of the four strains of legume bacteria was again roughly proportional to the quantity of nitrogen added in the soil extract. Practically no visible growths were secured on the media containing no soil extract, but fair to good growths were secured with the larger quantities of extract added. Duplicate nitrogen determinations checked unusually well, and the results, in general, show even more conclusively than in previous tables that the nodule bacteria did not fix atmospheric nitrogen. The variations from the control are within experimental error with about an equal number of negative results as positive. In this experiment the medium used was ideal for the growth of Rhizobia except for the absence of sufficient fixed nitrogen. Thin layers of solution and large flasks were used to provide excellent aerobic conditions, and yet no fixation was obtained with 4 representative strains of the nodule bacteria.

While the above experiment was in progress 5 additional cultures of root-nodule bacteria were received from E. B. Fred, of the University of Wisconsin, and a third experiment immediately started which was in all essential respects a duplication of that reported in Table 16. Again a soil extract was prepared, using a soil from which the nitrates were first leached with cold water. Two-liter flasks were used, some containing 100 c. c. and others 200 c. c. of the soil-extract medium, according to the scheme shown in Table 18. All tests were made in triplicate with each of the nine strains of Rhizobia. The first four of these were the cultures used in obtaining the results reported in Table 17, the other 5 were those received from Fred. After the cultures had been incubated for 41 days analyses were made by the Kjeldahl method, using mercury in the digestion flasks.

TABLE 18.—*Results of nitrogen determinations on cultures of Rhizobia grown on a mineral-sugar medium containing various amounts of soil extract and incubated 41 days*

Culture	Quantity of soil extract media	Growth observations	N	Average N	N fixed
	c. c.		*Mgm.*	*Mgm.*	*Mgm.*
Red clover	100	Good	1.97 2.04 2.03	2.01	−0.02
	200	do	3.82 3.82 3.84	3.83	.03
Cowpea	100	Fair	2.03 2.00 2.03	2.02	−.01
	200	do	3.84 3.82 3.78	3.81	.01
Soybean	100	do	2.06 lost 2.10	2.08	.05
	200	do	3.78 3.84 3.84	3.82	.02
Alfalfa	100	Slight	1.99 2.03 2.00	2.01	−.02
	200	Fair	3.82 3.81 3.78	3.80	.00
Clover (1)	100	do	2.14 2.00 2.03	2.06	.03
	200	Good	3.79 3.79 3.84	3.81	.01
Clover (2)	100	Fair	1.88 2.00 2.02	1.97	−.06
	200	Good	3.82 3.84 3.84	3.83	.03
Dalea	100	Fair	1.93 2.00 2.00	1.98	−.05
	200	Good	3.81 3.84 3.79	3.81	.01
Pea (1)	100	Fair	2.07 2.03 2.03	2.04	.01
	200	Good	3.82 3.84 3.82	3.83	.03
Pea (2)	100	Slight	1.96 2.00 2.04	2.00	−.03
	200	Good	3.81 3.84 3.79	3.81	.01
Control uninoculated	100		2.02 2.06 2.00	2.03	
	200		3.84 3.81 3.76	3.80	

The data agree very closely with those given in Table 17, and the conclusions to be drawn are essentially the same.

A fourth experiment, similar in many respects to those reported in Tables 17 and 18 was next started, using an extract of a soil from which the nitrates were not first leached with cold water. However, in order to make unnecessary the use of the reduction method to include nitrates, the soil used was secured from the field in the late winter when only a trace of nitrates was present. The medium contained Ashby's mineral solution with 0.05 per cent K_2HPO_4, 2 per cent dextrose, and 50 c. c. of soil extract. One hundred c. c. of this was used per 2-liter flask. To a portion of the flasks, as shown in Table 19, were added 750 mgm. agar. This provided for a comparison

of fixation, if any, on liquid and solid media under conditions which provided excellent aeration. The nine cultures of nodule bacteria used in the preceding experiment were also used in this. The analyses were made by the Kjeldahl method, using mercury as a catalyst. In addition, 18 liquid cultures were analyzed by the modified Kjeldahl method, using Devarda's alloy to reduce any nitrates present. This latter series was run largely as a check on the rest of the experiment. As a further check another series of cultures was used for nitrate determinations by the colorimetric method. The analyses made at the end of 53 days are given in Table 19.

TABLE 19.—*Results of nitrogen determinations on cultures of various strains of Rhizobia grown on modified Ashby's mineral solution, liquid and solid, containing soil extract and incubated 53 days*

Culture	Type of culture	Growth observations	Kjeldahl method			Modified Kjeldahl method		
			N	Average N	N fixed	N	Average N	N fixed
			Mgm.	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*
Red clover	Solution	Good	2.96 2.97 2.99	2.97	0.01	2.84 2.62	2.73	−0.23
	Agar	do	4.15 4.12 4.19	4.15	−.17			
Cowpea	Solution	Fair	2.97 2.93 2.93	2.94	−.02	2.77 2.73	2.75	−.21
	Agar	do	4.19 4.25 4.22	4.22	−.10			
Soybean	Solution	do	3.03 3.06 3.02	3.04	.08	2.62 2.77	2.70	−.26
	Agar	do	4.16 4.25 4.21	4.21	−.11			
Alfalfa	Solution	do	3.03 3.06 2.97	3.02	.06	2.96 3.03	3.00	.04
	Agar	Good	4.26 4.26 4.21	4.24	−.08			
Clover (1)	Solution	Fair	3.06 3.09 3.02	3.06	.10	2.89 2.98	2.94	−.02
	Agar	do	4.25 4.25 4.21	4.24	−.08			
Clover (2)	Solution	do	3.09 3.07 2.97	3.04	.08	2.75 2.72	2.74	−.22
	Agar	Good	4.26 4.39 4.19	4.28	−.04			
Dalea	Solution	do	2.93 lost 2.96	2.95	−.01	2.90 2.56	2.73	−.23
	Agar	do	4.12 4.11 4.37	4.20	−.12			
Pea (1)	Solution	do	2.93 2.88 2.88	2.90	−.06	2.69 2.91	2.80	−.16
	Agar	do	4.18 4.26 4.14	4.14	−.18			
Pea (2)	Solution	do	3.97 2.93 2.92	2.92	−.04	2.55 2.77	2.66	−.30
	Agar	do	2.90 3.97 4.09	4.00	−.32			
Control, uninoculated	Solution		3.93 2.86 2.88 2.82	2.86		2.94 2.98	2.96	
	Agar		2.88 4.28 4.26 4.29 4.18	4.25		4.36 4.34 4.27	4.32	

The results show a larger number of slight losses of nitrogen than of gains, but the variations from the control are not great enough to be significant. Certainly there is no evidence in these results to indicate that Rhizobium can fix nitrogen when grown on either a solid or liquid medium. Qualitative tests for nitrates in the cultures after the end of the growth period showed none present. This agrees with actual analyses by the two methods in showing that the use of the reduction method was not necessary in this case.

GENERAL DISCUSSION

A lengthy discussion of the data presented in the previous pages is entirely unnecessary since the results of all of the experiments agree in showing that the legume bacteria did not fix atmospheric nitrogen when grown under ordinary laboratory conditions independent of the host plant. Of course it would be foolish to say that these bacteria never fix nitrogen under any conditions because obviously no one can test all of the variables. The writer does feel, however, from the data presented that there is little justification for the more or less generally accepted idea that legume-nodule bacteria can fix small quantities of nitrogen when grown independent of the host.

As for the previous work done on this subject, little need be said that was not stated in the historical review. To summarize this phase of the subject, it may be stated that much of the earlier work was done with impure cultures and need not be considered. Furthermore, only a few investigators have made extensive studies of the subject and only a comparatively few papers present as many as 40 analyses. Rarely has an appreciable fixation been reported for cultures grown on nitrogen-free media, and often the more nitrogen added, the greater were the fixations obtained even where more nitrogen was added than the bacteria could possibly have used. The use of soil-extract media has also led to misleading results in some cases due to the presence of nitrate nitrogen. The "official" method using salicylic acid for the determination of total nitrogen, including nitrates, is reliable only in the absence of moisture. Bacteriologists have frequently used it for the determination of total nitrogen in soil extracts. The nitrogen not found by this method appears as "fixation" in the final analysis of the culture after the bacteria have converted the nitrates into proteins.

Since the evidence points very strongly to the fact that legume-nodule bacteria do not fix nitrogen outside of the nodule we naturally wonder if they fix nitrogen in the nodule. We must wait for additional studies for a definite answer to this question. Very likely the host plant is just as important as are the bacteria in the utilization of free nitrogen gas and both the higher and lower plants work in close cooperation in the fixation process.

SUMMARY

This paper presents a rather complete review with a discussion of the literature dealing with nitrogen fixation by legume-nodule bacteria grown independent of the host.

A report of a large number of experiments planned to determine whether legume bacteria possess the power of fixing nitrogen apart from the host is given. These investigations were conducted over a

period of five years, using 31 strains of legume-nodule bacteria, grown in a very large number of media and under a variety of cultural conditions. Practically all of the factors which have been reported as important in demonstrating fixation of nitrogen by these bacteria were given consideration. The experiments in no case gave any evidence that Rhizobia can fix atmospheric nitrogen when grown apart from the host. These findings are based on about 600 analyses of cultures reported in this paper, about 300 not reported here, and about 700 additional cultures not analyzed because they showed little or no growth on nitrogen-deficient media.

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EFFECT OF VARIOUS FUMIGANTS ON THE GERMINATION OF SEEDS [1]

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Recently several new substances have been developed as fumigants to replace carbon disulphide. In a series of recent publications, Roark and Cotton have discussed the merits of certain aliphatic chlorides,[2] ethylene dichloride,[3] certain alkyl and alkylene formates,[4] some esters of halogenated fatty acids,[5] and ethylene oxide.[6]

As some of these substances find commercial application as fumigants, notably ethylene dichloride and the formates, the question arises as to the extent to which they may affect the germination of seeds. In the work reported, Roark and Cotton tested the effect on the germination of wheat, but there was no evidence to show whether all seeds would react in the same way. Hoyt [7] reported the effect of ethylene dichloride on the germination of wheat, oats, beans, peanuts, and clover, dismissing as of no importance an increase of 24 per cent in the germination of peanuts and a decrease of 21 per cent in the germination of beans.

Although the most extensive use of fumigation is on wheat prior to milling, in which case the effect on germination is not important, the fumigating of seeds stored for the next season's planting is also practiced. In order to obtain further data concerning the effect of the new fumigants, the work here reported was undertaken. Six of the most promising fumigants were tested upon 13 different kinds of seeds, an attempt being made to select those most likely to be fumigated commercially.

Table 1 shows the minimum lethal concentration required to kill rice weevils (*Sitophylus oryza*) buried in wheat and exposed to the action of the fumigant for 24 hours. It shows also the boiling point and the specific gravity of the different fumigants tested. These data are compiled from the publications cited.

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[1] Received for publication Apr. 10, 1929; issued December, 1929.

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TABLE 1.—*Minimum lethal concentration, boiling point, and specific gravity of various fumigants tested*

[Rice weevils, buried in wheat were exposed to the fumigant for 24-hour periods]

Fumigant	Minimum lethal concentration	Boiling point	Specific gravity
	Mgm. per liter	*°C.*	
Ethylene oxide	30	10.7	0.89
Tertiary butyl chloride	34	51.0	.84
Isopropyl formate	53	71.3	.88
Methyl chloroacetate	73	131.5	1.22
Ethylene dichloride	226	83.7	1.26
Trichloroethylene	650	88.0	1.48

In carrying out the experiments reported in the present paper 100 gm. of the seed to be tested was placed in a 500 c. c. Erlenmeyer flask, and the fumigant, measured in a 1 c. c. pipette graduated to 0.01 c. c., was allowed to fall directly on the grain. The flasks were stoppered and allowed to stand for 24 hours at room temperature. Two concentrations were used, the minimum lethal concentration and twice that quantity. The results obtained are shown in Table 2.

TABLE 2.—*Percentage germination of different kinds of seeds when untreated and after a 24-hour exposure to various fumigants in different strengths* [a]

Kind of seed tested	Percentage germination of untreated seed	Percentage germination of seed after 24-hour exposure to—											
		Ethylene oxide		Tertiary butyl chloride		Isopropyl formate		Methyl chloroacetate		Ethylene dichloride		Trichloroethylene	
		30 mgm.	60 mgm.	34 mgm.	68 mgm.	53 mgm.	106 mgm.	73 mgm.	146 mgm.	226 mgm.	452 mgm.	650 mgm.	1,300 mgm.
Wheat	96	0	0	96	96	95	98	22	2	96	94	96	94
Oats	54	0	0	56	59	51	58	50	42	62	53	58	48
Barley	98	33	14	99	99	99	97	48	39	98	98	99	98
Rye	96	0	0	98	98	98	97	4	29	98	98	98	96
Corn	91	0	0	86	79	85	89	13	0	85	85	81	84
Buckwheat	67	0	0	67	70	63	64	56	60	62	64	65	56
Sunflower	93	12	0	90	88	92	92	92	65	92	92	96	90
Beans	91	34	30	93	93	90	92	67	63	86	93	91	93
Lima beans	84	0	0	90	84	86	90	76	45	87	88	82	86
Cowpeas	27	6	0	58	23	47	35	14	27	42	12	38	28
Alfalfa	64	58	57	63	59	60	66	62	66	59	72	60	64
Clover	32	10	4	36	34	26	38	30	24	35	32	31	36
Timothy	81	0	0	87	79	88	86	0	0	90	86	77	83

[a] The germination tests were made by the Seed Testing Laboratory, Bureau of Plant Industry; the tests were made in duplicate on 100 seeds.

Of the six fumigants tested, ethylene oxide and methyl chloroacetate were found to be injurious to the germination of grain. With the heavier concentration of ethylene oxide, nine of the varieties of seeds showed no germination whatever, and with the exception of alfalfa, the germination of the others was seriously impaired. The injury from methyl chloroacetate was not so severe, the stronger concentration reducing on an average the percentage of germination to approximately 50 per cent, alfalfa again being an outstanding exception.

The other four fumigants, tertiary butyl chloride, isopropyl formate, ethylene dichloride, and trichloroethylene, were all quite harmless. In no case did the average percentage germination of all 12 kinds of seeds (cowpeas being excluded) vary more than 2 per cent from that of the untreated seed, which was well within the limits of accuracy of the experiment.

The different seeds reacted rather uniformly, except cowpeas and alfalfa. The latter was by far the most resistant of all those tested. The percentage germination of the cowpeas, which was very low in the untreated seed, was stimulated to a remarkable degree by the weaker concentrations of the four fumigants that were not toxic, the average being 46, as against 27 for the untreated seed. It behaved on the whole so erratically and the seed was so poor, being badly infested with weevils, that it was omitted in making up the averages. Corn seemed to be affected somewhat unfavorably by both the weaker and the stronger concentrations of the nontoxic fumigants, its average germination being 84 per cent as against 91 per cent in the untreated seed.

CONCLUSIONS

Wheat, oats, barley, rye, corn, buckwheat, sunflower, beans, Lima beans, cowpeas, alfalfa, clover, and timothy seeds may be fumigated with tertiary butyl chloride, isopropyl formate, ethylene dichloride, and trichloroethylene in concentrations up to twice the minimum lethal concentration required to kill rice weevils without seriously impairing the germination of the seeds.

Ethylene oxide and methyl chloroacetate seriously impair the germination of these seeds.

RELATION OF STOMATAL BEHAVIOR TO STEM-RUST RESISTANCE IN WHEAT[1]

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INTRODUCTION

There has been much speculation regarding the nature of resistance of cereals to *Puccinia graminis* Pers. Such accurate observations and experiments as have been made indicate two types of resistance of wheat varieties to *Puccinia graminis tritici* Erikss. and Henn., namely, physiological and morphological.

The so-called physiological resistance appears to be due to some incompatibility between the wheat plant and the pathogene, but its exact nature is yet unknown. The germ tubes of the pathogene may enter physiologically resistant varieties in a perfectly normal manner, but after they have entered they kill some of the plant cells and then they themselves die. A variety, of course, may be resistant to certain physiologic forms of *Puccinia graminis tritici* and completely susceptible to others. There appears, therefore, to be a high degree of specificity between the host plant and particular physiologic forms. Physiologically resistant varieties may be resistant in certain regions in a given year and susceptible in other regions. Likewise they may be resistant in the same region in one year and susceptible in another. The amount of rust on them depends on the presence or absence of forms which can attack them. For example, Kanred wheat is nearly immune from about a dozen of the physiologic forms so far described and is completely susceptible to many of the others. There is nothing to indicate that Kanred is resistant for any other reason except that of a mutual antagonism between it and those particular physiologic forms to which it is resistant.

Varieties physiologically resistant to certain physiologic forms apparently are equally resistant in all stages of development. It was long supposed that varietal resistance could be explained entirely on the physiologic basis. But it was found that varieties might be susceptible in the seedling stage and at least moderately resistant in later stages of development. This led to the discovery of the morphological type of resistance.

Varieties with the morphological type of resistance may be physiologically susceptible, but there may be structural characteristics which prevent the rust fungus from developing extensively within the tissues. The pathogene may enter the host readily and can develop normally in the chlorophyllous tissues. In the stem the rust can

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grow only in the collenchyma bundles. If these are large and numerous, the organism can grow extensively and produce large pustules. In some varieties, however, there is so much sclerenchyma and the collenchyma bundles of the stem are so small and few that the rust is restricted to relatively small areas. The pustules, therefore, are correspondingly small, and for practical purposes the variety is partially resistant. Kota wheat, for example, is highly susceptible to certain physiologic forms of *Puccinia graminis tritici* in the seedling stage and at least moderately resistant in later stages. Therefore it often is resistant in the field. A variety, of course, may be physiologically susceptible to some physiologic forms, physiologically resistant to others, but morphologically resistant to both.

Still other varieties are susceptible when seedlings and older plants are inoculated and incubated artificially in the greenhouse, but they remain almost free from rust in the field despite the fact that physiologic forms to which they are susceptible in the greenhouse may be present in abundance in the field. When pustules do develop on these varieties in the field, however, they seem to be fairly normal. It appears, therefore, that they have neither physiological nor morphological resistance. It would seem that, although the rust can develop in these varieties when it once enters them, it often is prevented from entering. For example, Velvet Don (C. I.[3] 1445), a durum wheat, is very susceptible to form 21 of *Puccinia graminis tritici* when grown in the greenhouse. The fungus readily infects this variety in all stages of development, from the seedling stage until after the plants have headed. Velvet Don evidently has no physiological resistance to form 21. Furthermore, the development of the fungus does not seem to be restricted to any great extent by morphologic peculiarities of the host. The broad and deep collenchyma strands of the culm often coalesce to form wide bands extending lengthwise of the stem and about one-fourth or one-third of the distance around it. Apparently, then, Velvet Don has neither physiologic nor morphologic resistance. Nevertheless, there usually is little or no rust on this variety at University Farm, St. Paul, Minn., even in seasons when *Puccinia graminis tritici* form 21 is very prevalent. The same marked freedom from rust has been noted in other wheat varieties during certain seasons and in certain localities where considerable infection might be expected because of the presence of abundant inoculum and favorable environmental conditions for rust infection and development.

As the germ tubes of aeciospores and urediniospores of *Puccinia graminis tritici* enter wheat plants only through the stomata, it seems that stomatal conditions may account for the inability of the fungus to enter. The stomata are numerous on stem, leaf, rachis, glume, and awn of cereal plants. If they remain closed much of the time, however, it probably would be difficult for the germ tubes to enter under natural conditions in the field. It is quite likely also that the stomata of different varieties may behave quite differently. The writer therefore made a study of stomatal behavior in certain selected varieties of the factors affecting the stomatal movements, and the possible relation of stomatal behavior to the ability of germ tubes to enter.

[3] C. I. refers to accession number, Office of Cereal Crops and Diseases.

LITERATURE REVIEW

The relation of stomatal movement to infection of the sugar-beet plant by *Cercospora beticola* was studied by Pool and McKay (*11*)[4] in 1916. They found the greatest susceptibility to infection concomitant with the greatest stomatal activity, both occurring on leaves of the same degree of maturity. In detailed studies on germ-tube penetration they found that conidia may germinate and produce long germ tubes and yet not penetrate through closed stomata. Whenever infection occurred the stomata were open. The stomatal activity, in turn, was influenced by light, temperature, and relative humidity.

Cobb (*3*), as early as 1892, made some interesting observations on the number, size, and position of stomata on varieties of wheat and the relation of these to the entrance of the mycelium of stem rust. He noted (*v. 3, p. 199*) that the waxy bloom of glaucous varieties reduces the stomatal opening to "a crack so narrow that the promycelial threads of rust fail to enter it."

Hursh (*6*) investigated the number and size of stomata on wheat varieties and found no correlation with rust resistance. He suggested that the degree and duration of the stomatal opening are probably of more importance and greater influence in the entrance of germ tubes than either the number or the size of the stomata.

Allen (*2*), while studying the early stages of infection of *Puccinia triticina* on Little Club wheat, found that in material fixed in the morning many of the germ tubes had just entered the host and formed the substomatal vesicles. In the material fixed in the afternoon she found that infection was always more advanced and most of the substomatal vesicles had sent out infecting hyphae. She suggested that this daily rhythm might be due to the fact that the time of entrance is conditioned by the daily stomatal movements of the host, and that entrance waits upon natural opening of a stoma rather than upon mechanical force or chemical action.

Allen (*1*) studied the behavior of three physiologic forms of *Puccinia graminis tritici* on Khapli emmer and found that "the appressoria of all three forms of the fungus secrete some substance, which, if present in sufficient amount, penetrates and kills the guard cells and alters their walls. So far as can be judged by appearances, it is the same substance in all three, but varies in quantity. * * * On comparing the percentage of entries with the degree of stomatal injury, it is to be noted that where injuries are least serious the entries are greatest in number." In these cases the stomata probably closed as soon as the substance penetrated and killed the guard cells, for any outside influence which affects the guard cells and decreases their turgor tends to close the opening between them. The indications are that the stomata must open and remain open for a time, in order that the rust fungus may reach the interior of its host.

EXPERIMENTAL METHODS

The stomatal movements of several wheat varieties were studied in the uniform rust nursery at St. Paul, Minn., to determine, in a rather general way and under natural conditions, the influence of environmental factors on stomatal behavior of different varieties.

[4] Reference is made by number (italic) to "Literature cited," p. 947.

The field observations were supplemented by greenhouse studies in an attempt to control stomatal movements and to ascertain whether germ tubes of the fungus were able to enter susceptible wheat seedlings through closed stomata.

The stomata were observed directly under the low-power (16-mm.) objective of a standard microscope, a method suggested by Lloyd (*8*). This was to obviate the difficulties encountered when the epidermis is stripped from the plant and plunged into absolute alcohol as a fixative (*6*, *7*, *9*). After a little practice it is very easy to draw the plant part to be examined across the microscope stage and to focus on the stomata. Under favorable conditions the movements may be followed very closely for a time. However, in the present work the observations were made as quickly as possible, in order that the stomata might not change as a result of the disturbance of the plant under observation. The individual openings were not measured, but the condition of the majority of the stomata in several microscopic fields on certain plant parts was determined and taken as typical for that particular wheat variety. Four classes were arbitrarily established to express the degree of stomatal opening: Wide open, approximately half open, narrow slits, and closed. (Fig. 1.) Of course, the shape and structure of the stomata vary somewhat for the different cereal varieties, but in general the four established classes served the purpose very well. If the stomata were wide open or half open, the germ tubes of the fungus could enter easily. If the stomata were closed, the fungus was kept outside the host; and if the openings were narrow slits, it was assumed that the fungus was kept out or else entered with difficulty and by the exertion of mechanical force.

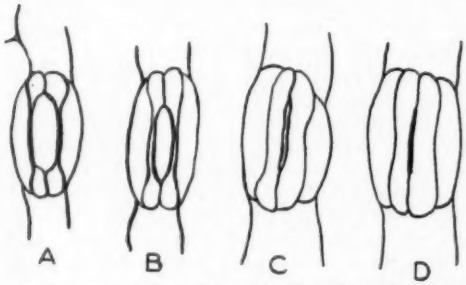

FIGURE 1.—A diagrammatic sketch giving the standards used in the determination of stomatal openings: A, Wide open; B, half open; C, narrow slits; D, closed

The following wheat varieties were studied in the field: *Triticum vulgare* (Little Club, C. I. 4066; Quality, C. I. 6607; Marquis, C. I. 3641; Baart (Early Baart) C. I. 1697; Haynes Bluestem, C. I. 2874; Reward, C. I. 8182; Ruby, C. I. 6047; Kota, C. I. 5878; Ceres, C. I. 6900; Marquillo, C. I. 6887; Webster, C. I. 3780; Hope, C. I. 8178); *Triticum durum* (Mindum, C. I. 5296; Arnautka, C. I. 1493; Kubanka, C. I. 2094; Acme, C. I. 5284; Velvet Don, C. I. 1445); and *Triticum dicoccum* (Khapli emmer, C. I. 4013 and Vernal emmer, C. I. 3686).

They were selected from the standard variety rows of the uniform rust nursery at St. Paul. Little Club and Quality were chosen as the two wheats which are extremely susceptible to stem rust whenever they are grown at St. Paul. Marquis, Baart (Early Baart), Haynes Bluestem, Reward, and Ruby usually are heavily rusted at St. Paul unless conditions are particularly unfavorable for the development of

infection. Mindum and Arnautka are very susceptible to most of the rust forms which attack the durums.

Infection on Kota and Ceres varies greatly from season to season, but even when the percentage of infection is high the type of infection is always the resistant one, with small limited pustules and signs of chlorosis and necrosis in some cases. Kubanka and Vernal emmer are usually resistant in the field also. Marquillo, Webster, Hope, Acme, Velvet Don, and Khapli emmer are always very resistant to stem rust at St. Paul. Very often the last-named varieties are almost free from rust.

The plants in the uniform rust nursery were sprayed repeatedly with spores of a large number of physiologic forms of stem rust, so that the chances for development of rust infection usually are greater there than in other sowings or in other localities.

STOMATAL BEHAVIOR IN THE FIELD

Many preliminary field observations were made under a wide variety of conditions before any definite conclusions could be drawn. The notes were taken during June and early July, from the time the plants were approaching the boot stage until after the heading of most varieties. During this time rust infection usually takes place. The inoculum probably is most abundant during the latter part of this period, but artificial epidemics are produced in the uniform rust nursery by spraying repeatedly with urediniospores of many physiologic forms of stem rust.

Loftfield (*9*) found a definite daily rhythm in the stomatal movements of cereals. Under favorable conditions the stomata open at sunrise, remain open during the morning, close gradually during the late afternoon, and remain closed all night. If conditions are unfavorable, most of the stomata remain closed during the day or are only partially open for an hour or so after sunrise. Night opening does not occur under ordinary conditions, favorable or unfavorable.

At first different parts of the plants were studied to see if the stomata of all parts behaved similarly. Loftfield (*9, p. 76*) found that the age and degree of maturity of a plant affected the behavior of the stomata and the readiness with which the stomata functioned. He found also that the degree of succulence of a plant affected the behavior of its stomata. Likewise, the age and the succulence of different plant parts might affect the behavior of stomata of those parts. A leaf blade of wheat is likely to be more succulent than the stem, and the young leaves more succulent than the older ones. On older, more mature plant parts many of the stomata function poorly or remain closed permanently, and the behavior of the stomata in a given area is not at all uniform.

In a particular variety of wheat the stomata of the youngest leaf respond to favorable stimuli more quickly than those of any other plant part. The stomata of the youngest leaf open first after sunrise. In the case of the older leaves the stomata respond to the sunlight a little less quickly perhaps; at any rate the stomatal behavior is less uniform, and most of the stomata lag somewhat in the opening process. Also, the stomata on the sheath of a plant, about midway between the first and the last node, lag a little behind the stomata of the young leaves; by the time the stomata of the young leaves have

opened wide those of the sheath may be only partly open. It may be that the light intensity is slightly lower and accounts for the somewhat slower opening of stomata of the sheath; or it may be merely the age of the sheath as compared with that of the leaf. The stomata on the neck or peduncle lag in opening even more than those on the sheath. They open more slowly and usually less widely than stomata of the young leaf. In this case there should be no decrease in light intensity to account for the slower response. The succulence of the stem tissue may be less than that of the young leaves, the relative humidity of the air about the heads and necks may be less than the

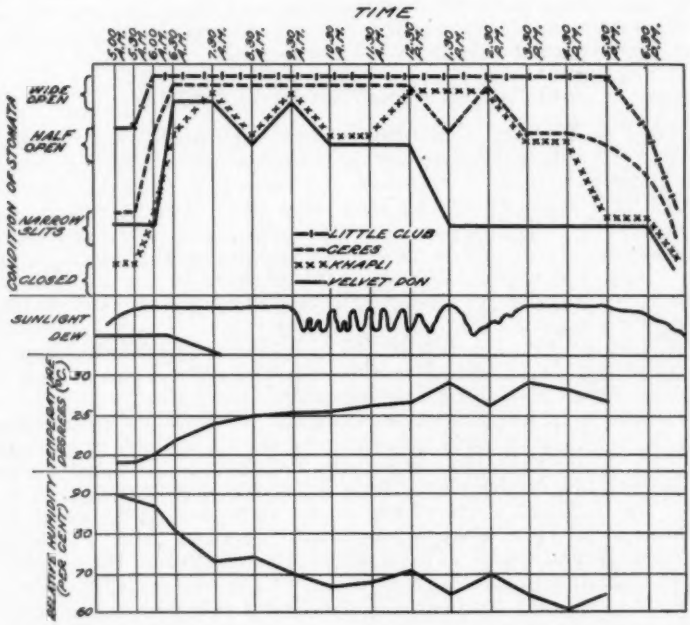

FIGURE 2.—The condition of stomata on four varieties of wheat in the field throughout a typical day during the first half of July at University Farm, St. Paul, Minn. The sunlight, dew, temperature, and relative humidity of the air were recorded at the time the stomata were observed

relative humidity about the lower parts of the plants, or there may be temperature differences in those regions; but it is impossible to say what causes the slight delay in opening of the stomata. The differences in stomatal behavior on different plant parts, although not great, are noticeable, particularly at certain periods of the day when the openings are changing rather rapidly. In all the field observations in which stomatal movements of wheat varieties were studied, care was taken that the comparisons were made between similar plant parts.

All the wheat varieties studied followed, in a general way, the trend of the daily rhythm in stomatal movements which Loftfield (*9*) observed in all cereals. However, varieties differed greatly in th e

details of stomatal behavior. The stomata of some varieties, Little Club, Baart (Early Baart), Quality, and Reward, usually opened almost immediately after sunrise, and under most conditions remained wide open until late in the afternoon. Stomata of these varieties respond very quickly to a definite, favorable stimulus, but they are not very sensitive to adverse changes in light, temperature, humidity, or to an increased transpiration of the plants. In other varieties, Marquis, Haynes Bluestem, Ceres, Ruby, Arnautka, and Mindum, the stomata usually opened a little more slowly in the early morning and were a little more sensitive to external conditions during the day. Under ordinary conditions, however, they remained wide open or partly open most of the day. The stomata of other varieties, Webster, Hope, Acme, and Velvet Don, opened very slowly in the morning and closed again rather early in the day unless conditions were very favorable for stomatal opening. Often, most of the stomata on these varieties remained closed during a large part of the day. Still other varieties, Kota, Marquillo, Khapli, and Kubanka, were intermediate with respect to the behavior of their stomata. Many observations were made under different environmental conditions, but it is impossible to give all the details here. Figure 2 shows the general trend of stomatal movements in four wheat varieties during a typical day early in July. The important point is that there are variations in stomatal behavior under the same environmental conditions, according to the variety of wheat observed.

With respect to the problem of rust infection, the behavior of stomata during the early morning hours immediately after sunrise is of the greatest importance. At that time the plants are covered with dew, so that, provided the temperature is not too low, conditions are ideal for the germination of rust spores lodged on the plants. If the stomata open early, a large proportion of the germinating spores effect entry before the dew is gone from the plants, and, once inside its host, the fungus develops according to its compatibility with the protoplasm of that particular host. The extent of its mycelial development and the formation of fruiting pustules depend to a large extent on the structure of the host (*6*), but the early stages of infection probably are almost entirely dependent upon the physiology of host and parasite.

A few selected wheat varieties were studied in the field so that some data might be obtained on stomatal movements during the critical hours of the early morning. The results of one series of observations are given in Figure 3. Six varieties of *Triticum vulgare* were chosen: Little Club and Quality, because they are extremely susceptible to most forms of stem rust; Marquis, because it usually is susceptible to a large number of rust forms; Kota, because its infection varies greatly under field conditions and it seems to be intermediate in its field resistance to stem rust; and Hope and Webster, because they usually are highly resistant to rust in the field, Hope being virtually free from infection in most cases. The first observations were made at 5.10 a. m., 20 minutes after sunrise. The stomata of Little Club and Quality were already partly open; those of Marquis appeared as narrow slits; and those of Hope, Webster, and Kota were still closed. The graph indicates the rapidity with which the stomata of each variety opened. A heavy dew was on the plants until 6.20 a. m., and then it gradually disappeared, so that at 8.20 a. m. the plants were

dry. If it is assumed that there was sufficient dew to protect the fungus germ tubes until about 7.30 a. m., it is found that there was approximately a 1-hour period during which the fungus might enter Hope and Webster plants, approximately 1⅓ hours during which Kota could be entered, 2 hours during which Marquis could be entered, and probably 2½ hours during which the fungus might enter Little Club and Quality. If the end of the heavy dew is taken as the limit for the survival of the germ tubes, then the periods of possible entry are considerably shortened; 1⅓ hours for the entry of Little Club and Quality, less than 1 hour for Marquis, about one-third of an hour for Kota, and no time for Webster and Hope because their stomata were not sufficiently open until the heavy dew had disappeared. At any rate, it is apparent that some wheat varieties are in a receptive

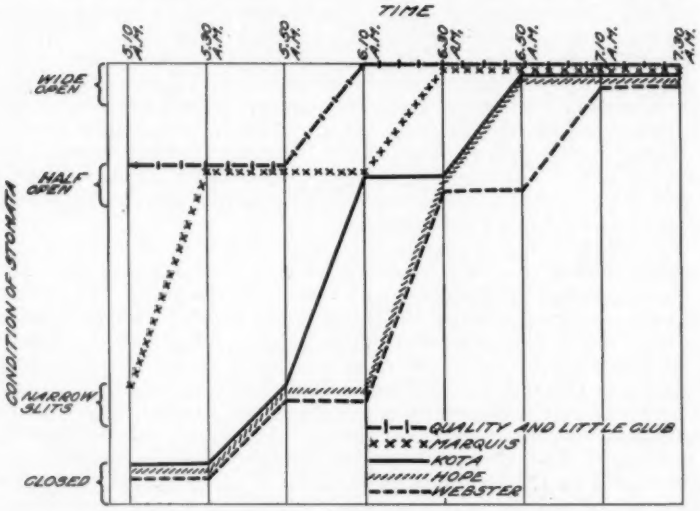

FIGURE 3.—The condition of stomata on wheat plants in the field during early morning hours. Observations started 20 minutes after sunrise on July 3, 1928, at University Farm, St. Paul, Minn.

state for longer periods than others. If similar conditions prevail throughout the growing period, it is easy to see that a large proportion of germinating rust spores will gain entrance to some varieties and may prove to be very effective inoculum, while on other varieties the great proportion of germinating rust spores will be excluded and only a relatively few germ tubes will enter and infect the host.

STOMATAL BEHAVIOR IN THE GREENHOUSE

Stomatal movements in Marquis wheat were studied in the greenhouse at University Farm during January, 1926. Later experiments included several other wheat varieties: Little Club, Kota, Webster, Hope, Ceres, and Marquillo of the *Triticum vulgare* group, and Khapli emmer. The same daily rhythm was noted in 1-week-old seedlings and in the older plants. Light seemed to be the most

important factor influencing the degree of opening, for during the winter months the stomata began to open between 7 and 8 a. m. and started to close between 3 and 4 p. m. Many attempts were made to prolong the period of opening, but very few were successful. Very young seedlings and older plants seemed to have established a daily rhythm in stomatal movement, which was scarcely to be altered by artificial light, humidity, or temperature.

THE EFFECT OF LIGHT

As the general trend of the stomatal movements in cereals seems closely correlated with sunlight, it was advisable to find out how quickly the stomata would respond to light in the greenhouse. For this purpose Little Club, Ceres, Marquillo, and Hope were grown in the greenhouse until they approached the boot stage. The plants were succulent and vigorous, and the stomatal movements followed the definite normal rhythm expected under favorable conditions. One series of observations was made during May, when the sun rose between 5.30 and 5.45 a. m. At 6 a. m. the sun was shining in one of the houses, while the other house was still shaded. The temperature in both houses was 20° C. One lot of plants was transferred from the shaded to the lighter house and placed directly in the rays of the sun. Although the sun was still low on the horizon and the slanting rays were not intense, there was a very decided response in the plants. Within 5 minutes after the transfer to direct sunlight, the stomata of leaves of Little Club started to open and most of them appeared as narrow slits. Within another 4 minutes most of the stomata of these leaves were half open; and after 20 minutes in direct sunlight nearly all were wide open. Ceres also responded quickly, though less so than Little Club. Within 10 minutes after the transfer some of the stomata on leaves of Ceres appeared as narrow slits, although many remained closed. Twenty minutes after the transfer some of the stomata of Ceres were half open, many appeared as narrow slits, and many others were still closed. One hour after the transfer most of the stomata of Ceres were partly open, but very few were wide open until later in the morning. Marquillo and Hope responded very slowly as compared with Little Club and Ceres, and it was easily noticeable that the stomata on very young leaves reacted more quickly than those on the older leaves. Twenty minutes after the transfer a few stomata on the leaves of Marquillo and Hope appeared as narrow slits, but by far the majority of stomata were closed. Even after one hour in direct sunlight many of the stomata on Marquillo and Hope were closed, some were in the narrow-slit stage, and only a very few were half open.

Parallel observations were made on the stomata of the plants left in the shaded house. At 7 o'clock, one hour after the first lot of plants had been transferred to direct sunlight, the stomata on leaves of Ceres and Little Club in the shaded house were just beginning to open and most of them appeared as narrow slits. The stomata on leaves of Marquillo and Hope in the shaded house were still tightly closed. The direct sunlight did not reach this house until about 9 o'clock.

It is evident that light is a very potent factor in stomatal behavior, and that stomata of some varieties of wheat may respond to the

stimulus of sunlight more rapidly than those of other varieties. The other conditions of the experiment were approximately the same. All the plants were at the same stage of development and all were vigorous and succulent. The temperature in the two greenhouses was the same (20° C.) at the beginning of the experiment, but there was a very gradual rise of temperature in the sunny house so that at 7 o'clock it was 23°. The relative humidity of the two houses varied from 70 to 75 per cent. Sunlight was the important variable in this experiment, and seems to account for the earlier opening of stomata on plants kept in the lighter house.

During the winter months a few experiments were made with artificial light. One-week-old seedlings of Little Club and Marquis wheats, which had been growing on the greenhouse bench and had established a normal daily rhythm in stomatal movements, were transferred to artificial-light rooms and placed about 2 feet below a 200-watt lamp. The transfer was made late in the afternoon, and when the plants on the greenhouse bench were examined just before the transfer the stomata were closing. It was hoped that the artificial light would cause the stomata to open again, but it did not. The plants were examined at intervals during the evening, but the stomata were closed. Check plants in the greenhouse were examined at the same intervals and their stomata were closed. During the period of observation the temperature of the light room was about 22° C., and the relative humidity was about 50 to 60 per cent; the temperature of the greenhouse varied from 16° to 19°, and the relative humidity was about 50 to 60 per cent. Repetitions of the experiment with seedlings, and also with older plants, gave the same results. Artificial light did not cause the stomata to open during the night after they had once closed.

In another experiment the conditions were varied slightly; the plants were transferred early in the afternoon of a bright sunshiny day, so that the stomata of the leaves were wide open at the time the plants were first exposed to artificial light. At 2.15 p. m. the temperature in the greenhouse was 25° C. and the relative humidity 60 per cent. The plants had been exposed to bright sunlight all day, and the stomata were wide open. One lot of plants was transferred to the light room as in the previous experiments, and another lot was left on the greenhouse bench. In the light room the temperature was 22° C. and the relative humidity 50 to 60 per cent. The first observations were made one hour after the transfer. In the light room most of the stomata were half open, but none were wide open. In the greenhouse the plants were shaded and the temperature had dropped to 22°. Most of the stomatal openings were merely narrow slits, although a few were half open. Observations were made hourly until 11 p. m. During that period the stomata of plants in the light room followed the normal rhythm, so that by 11 o'clock most of the stomata were closed and only a very few remained half open. The stomata on the plants in the greenhouse were all closed soon after sundown. Either the artificial light was not strong enough or the daily rhythm had become so well established that it could not be altered to any extent. Although some plants were kept in the light room for several days, their stomata did not remain open during the night.

THE EFFECT OF MOISTURE

Moisture and the relative humidity of the atmosphere have considerable influence on the stomatal movements of most green plants. Loftfield (*9, p. 75*) stated that a high atmospheric humidity permits stomata to open wider and remain open longer than a low humidity, and that when the leaves of a plant are wet by dew or rain or wet artificially the stomata usually open if closed, or open more widely if partially open.

Potted wheat plants were subjected to saturated atmospheres and to artificial wetting to determine whether the excessive moisture would prolong the period of stomatal opening after sunset. In the first experiment, made during the winter, 10-day-old seedlings of the varieties Little Club and Webster were placed under bell jars on the greenhouse bench. At 6 p. m., an hour or more after sunset, when the stomata were closed, one lot of seedlings was sprayed with water until the plants were thoroughly wet. A second lot was subjected to a saturated atmosphere under a bell jar without a direct spraying of the plants, and the third lot was left in the relatively dry air under a bell jar. The temperature in the greenhouse was about 12° C. throughout the evening. Stomata were observed again at 9 o'clock to see whether the excessive moisture had induced opening. All stomata in the three lots of plants were closed, so that moisture seemed to be ineffective in causing stomata to open after they had once closed. Similar experiments with plants approaching the boot stage of development gave similar results.

A second experiment was made to determine whether excessive moisture would cause open stomata to stay open longer than those of plants kept in relatively dry air. The experiment was started at 2 p. m. one clear January day. Khapli, Marquis, Kota, and Webster plants, all in the 4-leaf stage of development, had been on the greenhouse bench in a clear diffuse light. The stomata of the first three varieties were open, but those of Webster were gradually closing and were in the narrow-slit stage. The temperature was 23° C. at 2 p. m. The plants of one lot were sprayed until the leaves were thoroughly wet and were then placed in a saturated atmosphere under bell jars. Check plants were left on the greenhouse bench. The stomata were examined at 4.30 p. m., when the light was decreasing rapidly and the short winter day was drawing to a close. At that time the temperature in the greenhouse had dropped to 19°. All the stomata of the check plants and all those of the wetted plants in the saturated atmosphere under the bell jars were closed. The excessive moisture had not kept the stomata open.

One more experiment was made with very young wheat plants. Seeds of Mindum wheat were germinated in moist chambers, and before the green leaves appeared each seedling was placed in an individual moist chamber, so that the plants were exposed only to a saturated atmosphere from the beginning of their development. The very young green leaves were kept constantly wet and most of the stomata remained open or partly open after sunset and during the night. Observations were made at intervals, and conditions remained the same for 60 hours. At the end of that time the seedlings were removed, for they did not thrive under the close and excessively wet conditions of the moist chamber. Soon after the excess moisture evaporated from the leaf surfaces the stomata closed, and from then

on light seemed to be the most important factor influencing stomatal movements.

THE EFFECT OF TEMPERATURE AND MOISTURE

In many of the experiments to determine the effect of moisture on stomatal behavior, the temperature varied so much that it was decided to ascertain whether it modified the effect of moisture. There is a possibility that, if the temperature is favorable for stomatal opening, excessive moisture might cause the stomata to remain open at night.

The following experiment was made: Little Club and Webster wheats, in the 4-leaf stage, were kept at temperatures of 12°, 20°, and 27° C. during the day, and the stomatal movements were observed closely. The light was about the same in the three locations, and there was a normal rhythm in stomatal movements at all three temperatures. In the middle of the afternoon one lot of plants at each temperature was sprayed and placed in a saturated atmosphere under bell jars. Frequent examinations were made during the afternoon and evening, but all the stomata closed soon after sunset. Neither temperature nor moisture altered the normal behavior to any extent, for the stomata closed when the light stimulus was removed.

Inasmuch as the stomata of the cereals are closed during the night and sometimes for considerable periods during the day, it is doubtful whether the rust fungus is able to effect entry at all times.

ENTRANCE OF THE PARASITE THROUGH CLOSED STOMATA

It is essential to know whether the germ tubes of the stem-rust fungus normally enter a host plant through closed stomata. If they do so normally and easily, then stomatal behavior should have no effect on the amount of infection under conditions favorable for the development of rust.

This phase of the problem was studied in the greenhouse with seedling plants and with certain physiologic forms of stem rust to which most of the varieties of wheat used were very susceptible. Seedlings were used because the behavior of their stomata is more uniform over relatively large plant surfaces than that of stomata on older plants. Dust and other foreign particles often lodge between the guard cells on the older plants and keep the stomata open. Seedlings can be protected from such accidents and, moreover, can be handled more easily and quickly. The probability of successful infection when inoculations are made in the greenhouse seems to be greater with seedlings than with older plants. Inoculated seedlings are usually kept in the moist chamber for 48 hours after inoculation, but it is necessary that older plants be kept there for 72 hours or longer. Then again, the first pustules appear on seedlings within 7 to 10 days after inoculation, but with older plants the incubation period usually is longer. Thus, in an experiment of this kind, quicker and more reliable results should be obtained with seedlings than with older plants.

The first experiments were made with 3-day-old seedlings of Webster, Kota, and Little Club. The seeds were germinated in a moist chamber which protected them from dust and other foreign material. At the end of the third day, just before inoculation, the stomata were

examined, and as nearly as could be determined all were closed. The seedlings were atomized and inoculated with urediniospores of *Puccinia graminis tritici* form 17. They were then placed in moist chambers, which were kept in darkness so that the stomata would remain closed. Both Little Club and Kota are susceptible to form 17, giving 4 and 3+ reactions, respectively (*14*). Webster is moderately resistant to this form, the reaction varying from 2− to 3− (*15*). A check lot of seedlings was atomized and placed in another moist chamber for observation at intervals to see if the stomata remained closed. Spore-germination tests were made at the same time in order to be sure that effective inoculum was present. The inoculated seedlings were left in the dark moist chamber for 24 hours and then removed to open culture dishes, where they were supplied with Shive's solution (*12*) daily. Nine days after inoculation, single, minute uredinia appeared on the tips of two out of four Little Club seedlings and on one out of four Webster seedlings. The uredinia were so small as to indicate that a single germ tube had entered in each case. In the case of Little Club, the pustules were at the tips of the seedlings, and it was concluded that the fungus had entered through the open hydathodes at the tips of the leaves. In the case of Webster this was not true, for the pustule was located some distance behind the tip. The fungus either forced its way through a closed stoma or a few of the stomata were open during the time the plants were in the moist chamber.

In the next experiment greater precaution was taken to restrict the inoculation court. A second series of 4-day-old seedlings of Little Club, Kota, and Webster was inoculated with *Puccinia graminis tritici* form 17. The spores were carefully placed about one-half inch back of the leaf tip after the plants had been examined and the stomata found closed. One lot of each variety was placed in a moist chamber in the dark and left for 24 hours. Another lot was placed in moist chambers and left on a table where the light would reach them after 10 to 12 hours. At the end of 24 hours the plants were transferred to Shive's solution. Nine days after inoculation, two or three pustules appeared about 1 inch from the tips of each of the Kota seedlings which had been exposed to light 12 hours after inoculation. Infection seemed to be similar to that normally obtained in the greenhouse, except that the pustules were fewer. The Webster and Little Club plants of that lot did not thrive under the conditions of the experiment, and no pustules appeared during the first 12 days after inoculation. None of the plants kept in the dark moist chamber became infected during the 12 days.

Some time later, 24 Mindum seedlings were inoculated with *Puccinia graminis tritici* form 21, which produces a type 4− infection on this variety (*14*). The inoculum was placed about three-fourths of an inch from the tips of the seedlings to preclude the entry through hydathodes. The inoculated seedlings were atomized and placed in a moist chamber for six hours, during which time the stomata remained closed. In germination tests 80 per cent of the spores had germinated during the six hours and had produced germ tubes which would be long and vigorous enough to enter the host, unless they were prevented by the closed stomata. After six hours the seedlings were removed from the moist chambers, placed in culture jars, and supplied with

Shive's solution. Care was taken to prevent a collection of excess moisture on the leaves, in order that spores left on the surfaces might not germinate later and effect entry when the stomata were open. The seedlings grew well in the nutrient solution, and no signs of infection appeared during the first eight days following inoculation. On the ninth day very minute pustules appeared on 2 of the 24 seedlings, about three-fourths of an inch from the tips. The pustules were so very small that on first examination they seemed to be merely remains of the inoculum. Their development was very slow, and it was obvious that each pustule had originated from a single infecting germ tube. The inoculated plants were examined twice daily for 15 days. At the end of that time 20 seedlings were healthy and free from infection, 2 were free from infection but the tips of the leaves were shriveling, and 2 each had single minute rust pustules three-fourths of an inch from the leaf tip. The closed stomata apparently had excluded most of the germ tubes. A 6-hour period in the moist chamber seems a short time to permit infection, but it has been found that as short a period as three hours is sufficient for a considerable number of entries when the stomata are open.

The results of still another test are given in Table 1. While the numbers of seedlings used in this trial were very small, the results are very definite and indicative of the results obtained when larger numbers were used. On the seedlings inoculated at the leaf tips, where the hydathodes served as avenues of entrance, pustules appeared in from 5 to 7 days after inoculation, regardless of whether the seedlings had been in dark moist chambers or in those exposed to the light for a part of the time. On the seedlings inoculated three-fourths of an inch back of the tip, pustules appeared from 7 to 10 days after inoculation, but only on those which had been in moist chambers exposed to the light. No pustules appeared on seedlings kept in the dark moist chambers, probably because the stomata were closed while the rust spores were germinating and the germ tubes were in condition to enter the plant.

Sections were made of a few of the inoculated seedlings to see whether there was any evidence that the germ tubes had forced their way between the guard cells. Many of the germ tubes had formed appressoria and on some of them there were short projections which seemed to fit into the depression between the two guard cells although the stoma was closed. However, there was only one case which possibly might be interpreted as a forced entry. An appresorium was formed over a stoma, and in cross section a thick, wedge-shaped projection appeared to have forced the guard cells apart until the fungus had penetrated about half the depth of the stomatal slit. The inner half of the stoma was closed, and the walls of the guard cells were pressed tightly against each other. The fungus may have wedged its way into the stoma, or the stoma may have been partly open at the time the fungus reached it and may have closed again before the fungus could enter. The conclusions drawn from the prepared slides were that the rust fungus generally does not enter the host plant if the stomata are closed.

TABLE 1.—*The results of inoculating Little Club and Webster wheat seedlings with Puccinia graminis tritici form 17 at and near the leaf tips when the stomata were closed, and incubating them for 24 hours in moist chambers kept in darkness or exposed to light after 12 hours*

[The numerator of the fraction indicates the number of infected seedlings; the denominator, the total number of seedlings]

Variety used	Date of inoculation (1927)	Inoculation court	Moist chamber conditions	Infection at specified times after inoculation			
				5 days	7 days	8 days	10 days
Little Club	Dec. 5	Tip of seedling	Dark	2/3	3/3		
			Light	3/3	3/3		
Do	Dec. 7	¾ inch from tip	Dark		0/5	0/5	0/5
			Light		0/5	1/5	4/5
Webster	Dec. 5	Tip of seedling	Dark	0/2	2/2		
			Light	0/2	2/2		
Do	Dec. 7	¾ inch from tip	Dark		0/5	0/5	0/5
			Light		1/5	2/5	3/5

DISCUSSION

In the study of disease resistance, and especially the rust resistance of cereals, it has been impossible to find a universal explanation for the resistance of some varieties and the susceptibility of others. There apparently are several types of resistance to stem rust. Many investigators have studied the so-called physiologic resistance of certain cereals and the phenomena associated with infection of a resistant host by a rust fungus (*4*, *10*, *13*, *16*), and Hursh (*6*) has definitely shown that the morphology of the host may have a pronounced effect on the development of rust in certain varieties of wheat. The present writer has shown that there may be a third factor termed "functional resistance," which may enable some varieties to escape infection because of their characteristic stomatal behavior.

As the stomata of the cereals constitute the avenues of entrance for the germ tubes of rusts, and stem-rust germ tubes do not enter the host unless its stomata are open, the factors that influence the stomatal movements would indirectly influence the course of rust infection. The stomata of cereals are always closed at night, and remain so until sunrise or later. Hence, even though many urediniospores on the plant surfaces germinate during the night, few or no germ tubes can enter the host until the return of the sunlight causes the opening of stomata. During the growing period in the upper Mississippi Valley there usually are heavy dews which often remain on the plants for a considerable time after sunrise. Naturally, the dew remains longer on plants in the heavier stands of grain, but even on those in moderatly light stands the plants are wet for some time after sunrise. This provides an ideal condition for the germination of rust spores and for the survival of germ tubes which may have been formed during the night. By far the greatest number of infections occur during this critical period between sunrise and the disappearance of the dew. The stomatal behavior of the cereal hosts enters into the problem at this time.

If the stomata of a particular variety open quickly in response to the stimulus of sunlight, it is obvious that the germ tubes may enter sooner and that the period favorable for entry is longer than in the case of another variety whose stomata respond very slowly and do not open for an hour or more after sunrise. After the dew disappears and the surfaces of the plants become dry, most of the delicate fungus germ tubes die, unless they have grown into the plant. If most of the stomata of the plant remain closed until after the dew disappears, most of the germ tubes will perish and never enter the host. Stomatal movements probably vary greatly under different environmental conditions, but it seems highly probable that under ordinary conditions in the upper Mississippi Valley the stomatal behavior of some wheat varieties usually is such as to exclude a large number of the stem-rust germ tubes.

For many years certain varieties of wheat grown at University Farm, St. Paul, Minn., have been consistently resistant to stem rust when grown in the field, in spite of the fact that they are susceptible to some forms of *Puccinia graminis tritici* when inoculated in the seedling stage in the greenhouse. Rust surveys have shown that forms of rust which can attack these varieties are present in the field. Although there should be plenty of inoculum, little or no rust develops on them. Why is it? Is there, as some workers have suggested, a "mature plant resistance" which is not evident in the seedling stages but which develops later in the life of the plant? Or can some of this resistance be explained on the basis of stomatal behavior of the wheat variety in question?

Kota is very often resistant to stem rust in the field at University Farm. The infection varies from a trace, as in 1919, 1921, and 1922, to 12 to 20 per cent, as in 1925 and 1927, or even as high as 33 per cent, as in 1920. Hursh (*6*) has shown that the structure of Kota limits the size of the rust pustules after the fungus enters the host tissues, but in many seasons Kota is almost free from rust, and there is so little evidence of infection and subsequent pustule formation as to indicate complete exclusion from the host. Likewise, the amount of infection on Kota varies according to locality. In some of the uniform rust nurseries the percentage of infection is relatively high (25 to 35 per cent at Madison, Wis., and 38 to 75 per cent at Morris, Minn., in 1927), while in other nurseries only a small amount of rust develops (1 to 2 per cent at Manhattan, Kans., and 4 to 10 per cent at Fargo, N. Dak., in 1927). Forms of stem rust to which Kota is susceptible may be present in all of the nurseries (forms 18 and 21 of *Puccinia graminis tritici* were isolated from Madison, Morris Manhattan, and Fargo, in 1927), but the percentage of infection varies greatly.[5] It is probable that stomatal behavior may be partly responsible for some of these differences.

In the field there is great variation in the amount of stem rust on Webster wheat, which is moderately resistant to those forms of *Puccinia graminis tritici* with which it has been inoculated in the greenhouse. In the uniform rust nursery at University Farm, only 5 to 10 per cent of stem rust developed on Webster. On the sandy experimental plots at Coon Creek, Minn., there was 63 to 90 per cent

[5] Unpublished results of Stakman and his coworkers, made available through the kindness of Dr. E. C. Stakman.

infection on Webster in 1927, although the pustules were all relatively small and the majority of them never ruptured the epidermis. Stomatal behavior was not studied at Coon Creek, but it is certain that a great many more entries were effected there than at University Farm.

Velvet Don has been grown in the field at University Farm since 1909, and during that time severe epidemics of stem rust were produced artificially. During the whole 19-year period, however, Velvet Don has always been very resistant and usually not more than a trace to 5 or 10 per cent of stem rust develops. When seedlings of this variety are inoculated in the greenhouse with *Puccinia graminis tritici* form 21, a type 4 infection results, indicating marked susceptibility to that form. When greenhouse inoculations are made later, on plants in the 4-leaf stage, in the boot, or after heading, excellent infection results, an abundance of rust pustules develop, and there is no indication of resistance. What accounts for the marked resistance in the field? The host grows vigorously and should be in suitable condition for infection; the virulent rust forms are present almost every year; but there is very little infection. Preliminary observations have been made on stomata of Velvet Don, and it may be that stomatal behavior accounts for a large part of the field resistance at University Farm. In the greenhouse the older plants are kept in the moist chamber for three days after inoculation, and in that period there probably is considerable time during which the stomata are open and the germinating spores are entering the plants. In the field the periods of stomatal opening probably do not coincide with the periods favorable for spore germination and growth of germ tubes, so that the fungus seldom gets inside the plants of this variety.

There is evidence also that in some years a great number of stem-rust germ tubes enter Khapli emmer grown in the field at University Farm. Khapli has a marked physiological resistance to all the forms of stem rust so far found in the United States, but it is a fact that, under favorable conditions, *Puccinia graminis tritici* enters such a resistant host as readily as it enters a highly susceptible host. The development of the mycelium is never very extensive in Khapli, and fruiting pustules usually are not formed. A cursory field examination of Khapli reveals no evidence of infection, but if the stem of the plant is drawn between the fingers the surface feels uneven and bumpy, owing to the distension of the epidermis over tissues infected by the rust. A microscopic examination of a cross section of the stem shows that the fungus has entered the host, and its mycelium has developed in the chlorophyllous strands of the wheat to such an extent that the epidermis is streched and distended without being ruptured. In some years this type of infection is very usual on Khapli; in other years Khapli is almost entirely free from rust, the stems are smooth and even, and apparently the fungus has been excluded from the plants. It seems probable that the stomatal behavior of Khapli is an important factor in the exclusion of the rust in these latter cases.

Goulden, Neatby, and Welsh (*5*) investigated the "mature plant resistance" in a cross between Marquis and H–44–24. They studied the inheritance of field resistance and concluded that it could be explained on a single-factor basis. Although this particular problem

has not been studied, it is possible that the single factor may be stomatal behavior. The present studies indicate that such a physiological tendency or habit is inherited in the same way as are the factors for morphologic and physiologic characters in general.

Stomatal behavior of the cereals evidently is far more important in the phenomena of rust infection than has been recognized heretofore, and it should be studied in greater detail. Stomatal behavior may be a more effectual means of resistance to stem rust than a fundamental protoplasmic resistance or even a morphological resistance. A variety which excludes the rust by its stomatal behavior would be resistant to all rust forms, not merely to some of them. Therefore, the problem of producing satisfactory rust-resistant varieties may be simpler than it has appeared to be.

SUMMARY

Two kinds of resistance to stem rust have been demonstrated previously: Physiological resistance and morphological resistance. To these may be added a third kind, termed "functional resistance."

Some varieties of wheat are resistant to stem rust in the field because of the behavior of their stomata. Such varieties may be susceptible to stem rust in the seedling stages of their development.

Stomatal movements of wheats follow a definite daily rhythm. The stomata open gradually after sunrise, remain open for varying lengths of time, close gradually during the afternoon, and remain closed all night. The daily rhythm of stomatal movements differs considerably in different varieties of wheat. Stomata of some varieties open very soon after sunrise and usually remain open most of the day. Stomata of other varieties open very slowly and remain open only a short time. There also are varieties with an intermediate type of stomatal behavior.

Stomata of the younger and more succulent plant parts open sooner and remain open longer than stomata on older or less succulent parts. Stomata on the young wheat leaves open sooner than most of the stomata on the older leaves, on the sheath, and on the peduncle of the plant. The differences are not great but are appreciable under ordinary conditions.

The critical period for stem-rust infection is in the early morning immediately after sunrise and while the plants are heavy with dew. The fungus easily enters its host if the stomata are open during most of the critical period, but if they are closed during that time the fungus is excluded. There are great differences in the stomatal behavior of some wheat varieties during this critical period.

Direct sunlight seems to be the most important stimulus for the opening of stomata of cereals.

Artificial light, excess of moisture, and excess of moisture and temperature combined did not prolong the period of openness for stomata of cereals in the greenhouse.

From inoculation and histological studies it appears that the stem-rust germ tubes generally, and probably always, enter the host only when the stomata are open. The fungus does not seem to force its way through closed stomata.

A variety of wheat may appear truly resistant to stem rust if the behavior of its stomata is such that most of the inoculum is excluded and rendered ineffective.

There is a correlation between stomatal behavior and the resistance of certain varieties of wheat to stem rust in the field at University Farm, St. Paul. Stomata of the highly susceptible varieties, Little Club, Baart (Early Baart), Quality, and Reward, open soon after sunrise and remain open most of the day. Stomata of some slightly less susceptible varieties, Marquis, Ruby, Haynes Bluestem, Arnautka and Mindum, open a little more slowly after sunrise, but they too remain open most of the day. In the varieties which are highly resistant in the field, Hope, Webster, Acme, and Velvet Don, the stomata open very slowly and close again relatively early in the day. Varieties which are moderately resistant in the field, Kota and Kubanka, have an intermediate type of stomatal behavior.

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THE MINERAL CONTENT OF THE JUJUBE[1]

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INTRODUCTION

Since the jujube or Chinese date (*Zizyphus jujuba* Mill.) promises to become a satisfactory tree for southern cultivation, the chemical composition of its fruit begins to assume importance. Church[2] has analyzed the pulp of several varieties and has made comparisons between its composition and that of the fig and date. Table 1, giving some data taken from Table 1 of his article, shows the composition of the edible matter in the fruit of S. P. I. No. 30488, a variety used in the present study.

TABLE 1.—*Percentage composition of the edible material of jujubes on a moisture-free basis*

S. P. I. No.	Sugars			Acid as anhydrous citric	Protein N×6.25	Crude fiber	Ash	Undetermined
	Reducing	Sucrose	Total					
30488 [a]	35.50	37.86	73.36	0.80	2.73	3.46	1.90	17.77
30488 [b]	30.73	37.90	68.63	.80	3.41	3.41	1.97	21.76

[a] Was fully colored. [b] Partially colored. Both were partly wrinkled and soft.

A search of the literature has revealed no data on the mineral content of the jujube, and it was therefore thought advisable to make a mineral analysis. The fruit was collected in the fall of 1928 from two trees, of the varieties designated as S. P. I. Nos. 17752 and 30488, which have been growing in the experiment station orchard since 1917. The fruit was in approximately the same state as Church's first sample in Table 1, fully colored and quite wrinkled.

EXPERIMENTAL PROCEDURE

The fruit was washed, thoroughly scrubbed with a small brush, and dried in the sun; the pulp was separated from the seeds as completely as possible by stripping and scraping off with a knife, and seeds and pulp were dried separately (1) for several days at 70° C. and (2) overnight at 110°. The dried seeds with adhering pulp fragments were then rubbed gently in a mortar, and the powder which separated was added to the dried pulp. The material was then ground, thoroughly sifted, and placed in open beakers in a large desiccator, where it stood for some time before analytical work began. Material intended for

[1] Received for publication May 11, 1929; issued December, 1929. Published with the permission of the director of the experiment station.

[2] THOMAS, C. C. THE CHINESE JUJUBE. U. S. Dept. Agr. Bul. 1215, 31 p., illus. 1924. (With a chapter on Composition of the Chinese Jujube, by C. G. Church, p. 24-29.)

iron analyses was ground in a porcelain mortar; for the rest a Wiley mill was used. The dried material was kept in the desiccator between times when samples were being taken. Occasionally total-ash determinations were made in order to detect any change in composition.

The analytical methods used were among those recommended by the Association of Official Agricultural Chemists [3] except that the potassium was weighed as the perchlorate, and an adaptation of Kennedy's [4] method was used for iron.

Iron determinations were carried out as follows: 5 gm. of dried material was ashed at a dull-red heat to a gray ash, treated with concentrated HNO_3, and reashed; the process being repeated, if necessary, till no carbon particles remained. The residue was digested with 2 c. c. of concentrated HCl and 3 drops of concentrated HNO_3. The solution and the insoluble silica were washed into a 50 c. c. flask and diluted to the mark. In the case of the raisins and dates, which were analyzed along with the jujubes for purposes of comparison, 4 c. c. of concentrated HCl was used and the sample diluted to 100 c. c. Ten cubic centimeter portions were pipetted into glass-stoppered bottles, treated with 10 c. c. of amyl alcohol and 5 c. c. of 20 per cent KCNS solution, the mixture shaken gently, and the alcohol layer pipetted into a colorimeter cup, and the color compared with that of a standard. A standard was made up with analytical iron wire dissolved in H_2SO_4 and oxidized with a little HNO_3. From the first standard, others of convenient concentrations were prepared as follows: A measured volume of the iron solution was placed in a graduated flask, a measured quantity of 1:4 HCl was added, and the flask filled to the mark with distilled water. Ten cubic centimeter portions of the iron solutions of the fruits had been titrated with 0.2 N NaOH; and the amount of 1:4 HCl added in preparing the standards was the calculated amount required to make the acidity, volume for volume, of the standards equal to the average acidity of the solutions under examination.

The presence of interfering substances, phosphates and the like, was tested for by making equal mixtures of the standard and the solutions under examination and comparing these with the standard in the colorimeter. The observed iron content of such mixtures in every case compared closely enough with the calculated value to indicate that interfering compounds could not be present in significant amounts.

The raisins and dates used were of standard brands, bought on the market.

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[3] ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS. OFFICIAL AND TENTATIVE METHODS OF ANALYSIS. COMPILED BY THE COMMITTEE ON EDITING OF ANALYSIS. REVISED TO JULY 1, 1924, Ed. 2, 535 p., illus. Washington, D. C. 1925.

[4] KENNEDY, R. P. THE QUANTITATIVE DETERMINATION OF IRON IN TISSUES. Jour. Biol. Chem. 385-391, illus. 1927.

ANALYTICAL RESULTS

The results of the analyses are shown in Tables 2 and 3.

TABLE 2.—*Percentage of ash and individual mineral constituents in jujubes as compared with dates and seeded raisins*

Fruit	Ash [a] in edible material, original basis	Ash in edible material, moisture-free basis	Mineral constituents on a moisture-free basis									
			CaO	MgO	K_2O	Na_2O	P_2O_5	MnO	Fe_2O_3	SO_3	Cl	SiO_2 soluble
Date	1.46	2.04	0.138	0.138	0.964	0.079	0.016	.....	0.0096	0.149	0.412	0.122
Seeded raisin	2.04	2.94	.078	.088	1.445	.122	0.031	.....	.0169	.118	.040	.171
Jujube S. P. I. No. 17752	1.03	1.84	.100	.083	1.056	.031	.019	0.021	.0021	.034	.076	.005
Jujube S. P. I. No. 30488	1.42	2.36	.132	.083	1.318	.033	.015	.023	.0027	.033	.086	.006

[a] For this determination, raisins and dates were used as they came from the package, jujubes after being cleaned and dried for several days in the sun.

TABLE 3.—*Composition of ash of jujubes compared with that of dates and seeded raisins*

Fruit	Total ash on a moisture-free basis	Percentage composition of ash									
		CaO	MgO	K_2O	Na_2O	P_2O_5	MnO	Fe_2O_3	SO_3	Cl	SiO_2 (soluble)
	Per cent										
Date	2.04	6.63	6.52	45.04	2.36	0.78	.....	0.471	7.35	19.06	5.77
Seeded raisin	2.94	2.64	2.97	48.72	4.11	1.06	.....	.576	4.01	1.31	4.81
Jujube S. P. I. No. 17752	1.84	5.42	4.46	56.40	1.65	1.04	1.11	.115	1.85	4.08	.28
Jujube S. P. I. No. 30488	2.36	5.60	3.53	54.78	1.29	.64	.99	.115	1.40	3.49	.27

Considering those minerals which are of chief nutritional significance, the two varieties of the jujube studied are seen to compare favorably with the date and raisin as sources of phosphorus and calcium but to be markedly inferior in iron content.

TRANSGRESSIVE SEGREGATION FOR SUSCEPTIBILITY TO SMUT IN AN OAT CROSS[1]

By R. J. GARBER, N. J. GIDDINGS, and M. M. HOOVER, *West Virginia Agricultural Experiment Station*

INTRODUCTION

In a recent paper [2] evidence of transgressive segregation for susceptibility to smut in the oat cross Gopher×Black Mesdag was presented. During the season of 1928 further data relative to this case were collected, and it is the purpose of the present paper to present some of these.

The pertinent literature was reviewed, and a description of the general methods followed in this investigation was given in the article cited above. During the season of 1928 the F_3 families were grown in triplicate plots, but the F_4 and F_5 families were grown in duplicate plots. In all, there were planted 120 seeds of each F_3 family, 80 seeds of each F_4 and F_5 family, and 40 seeds in each parental plot. The parents were interspersed at intervals of 10 plots (20 rows) among the progeny. Each plot in the nursery consisted of two rows 5 feet long. Consequently, with but 20 seeds planted per row, there was ample space for individual plant development.

All seed was treated with smut [3] spores about one month before the date of planting (June 16 and 18). A fairly successful smut epidemic was obtained, although in certain places in the nursery there seemed to be a somewhat lower infection than in others, as indicated by the degree of infection of the Gopher parent. Smut notes were taken after all the oats were completely headed and again at harvest time.

F_3 FAMILIES

The percentage of smutted plants and the color of seed of the F_3 families and of the parent stocks grown in 1928 as well as those already reported [4] are shown in Table 1. Of the 50 F_3 families grown in 1928, 13 showed no evidence of smut infection and 6 gave rather striking evidence of a susceptibility to smut greater than that of the Gopher parent. The other 31 F_3 families showed a range in percentage of smut infection which fell within that of the susceptible parent. It is apparent that the data collected in 1928 are similar to those obtained in 1926. In the latter year each F_3 family was grown in a single plot of three rows only, which accounts in part at least for the somewhat greater variability obtained. Each variate recorded in the frequency distributions for 1926 represents a single plot, whereas each variate recorded for 1928 represents triplicate

[1] Received for publication Feb. 19, 1929; issued December, 1929. Approved as Scientific Paper No. 72 by the director, West Virginia Agricultural Experiment Station.

[2] GARBER, R. J., GIDDINGS, N. J., and HOOVER, M. M. BREEDING FOR DISEASE RESISTANCE WITH PARTICULAR REFERENCE TO THE SMUT OF OATS. Sci. Agr. 9: 103–115. 1928.

[3] No effort was made to distinguish between loose smut (*Ustilago avenae*) and covered smut (*U. levis*), although most of the oat smut which occurs naturally in the vicinity of Morgantown, W. Va., is *U. avenae*

[4] GARBER, R. J., GIDDINGS, N. J., and HOOVER, M. M. Op. cit.

plots. On the basis of progeny tests it was found that 2 F_3 families recorded as "zero infection" in 1926 were wrongly classified. The correction has been made in Table 1.

Of the 150 F_3 families which were grown, 32 showed no evidence of smut infection and 13 showed transgressive segregation for smut susceptibility. Assuming that a single main factor difference is responsible for determining the high degree of resistance, if not immunity, of the Black Mesdag parent, one would expect one-fourth, or 37.5, of the F_3 families to be similar to it in smut reaction, whereas 32 actually were obtained. The difference (5.5 ± 3.6) is not significant.

TABLE 1.—*Number of smutted plants in various percentage classes and color of seed among the parents and F_3 families of an oat cross, Gopher×Black Mesdag, and the reciprocal, grown in 1926 and in 1928, at Morgantown, W. Va.*

Parent or progeny	Year grown	Color of seed	Number of plants in smut-percentage classes															Total
			0	2.5	7.5	12.5	17.5	22.5	27.5	32.5	37.5	42.5	47.5	52.5	57.5	62.5	67.5	
Gopher	1926	White	1	1	1	1		3	2	1	1							11
Black Mesdag	1926	Black	11															11
F_3 families	1926	do	7	6	5	3		1	1			1		1			1	26
Do	1926	Segregating	7	8	19	9			2	2	1				3			51
Do	1926	White	5	6	1	4		2	1	1	2	1						23
Gopher	1928	do		2	2	1												5
Black Mesdag	1928	Black	5															5
F_3 families	1928	do	4	5	2	1		1										13
Do	1928	Segregating	7	6	7	1		2	2	1								26
Do	1928	White	2	2	6	1												11

The F_3 families which showed a susceptibility to smut greater than that of the Gopher parent indicate that at least one additional hereditary factor is conditioning smut reaction in this cross. The Gopher parent has consistently proved to be of moderate susceptibility as compared with the highly susceptible progeny lines.

LINKAGE

Some evidence of a genetic linkage between the black-color gene and at least one factor which is causing increased smut susceptibility has been published.[5] The data obtained in 1928 corroborate those obtained earlier. The six F_3 families which showed transgressive segregation for smut susceptibility were either black seeded or segregated for black-seed color. In 1926 there were also six F_3 families which showed susceptibility to smut clearly beyond the range of the Gopher parent and two additional families were doubtful but showed percentages of infection a little greater than that of the most susceptible plot of Gopher. One of these F_3 families had white seed and the other black.

F_4 FAMILIES

The F_4 families grown in 1927, and already reported, are shown in Table 2. Family 17–10–30 did not show any smut infection in F_3, but one of the five F_4 families grown from it had a single smutted plant. Further tests in F_5 indicated that this family was truly a

[5] GARBER, R. J., GIDDINGS, N. J., and HOOVER, M. M., Op. cit.

zero-infection strain; hence a correction has been made in the data originally published.

All of the families except one noted as zero infection in F_3 apparently bred true for this character in F_4. One F_3 family, 16–15–59, produced four F_4 families which showed smut to one which did not; consequently the F_3 should probably have been classed as a low-infection rather than as a zero-infection strain.

TABLE 2.—*Percentage of smutted plants among the F_3 families, number of smutted plants in various percentage classes among the respective F_4 families, and the parents grown among the latter, of an oat cross, Gopher×Black Mesdag, and the reciprocal, grown in 1927.*

Parent or progeny	F_3 families		Number of plants in various smut-percentage classes among F_4 families										Total [a]
	Plants	Smutted	0	2.5	7.5	12.5	17.5	22.5	27.5	32.5	37.5	42.5	
	Number	*Per cent*											
Gopher				3	7	5		1					16
Black Mesdag			16										16
16-15-4	64	0	5										5
16-15-8	66	1.5	3	1		1							5
16-15-7	64	12.5	1	3	1								5
16-15-39	60	55.0				2	2		1				5
16-15-16	70	0	5										5
16-15-31	66	1.5	2	3									5
16-15-1	51	13.7	2	1	2								5
16-15-21	70	27.1			3			1	1				5
16-15-17	70	0	8										8
16-15-35	63	0	5										5
16-15-36	54	20.4	2	1	1	1							5
16-15-53	69	0	5										5
16-15-55	70	27.1		1	1	1	1		1				5
16-15-59	65	0	1	3	1								5
16-15-64	60	0	5										5
16-15-68	73	28.8		2		3							5
17-10-1	68	0	5										5
17-10-21	65	56.9				2	1	1	1				5
17-10-22	65	1.5	1	2	1	1							5
17-10-35	65	27.7	1		1	2	1						5
17-10-30	61	0	5										5
17-10-54	63	11.1	3	1		1							5
17-10-62	67	0	5										5
17-10-64	67	50.7				2	1	1	1				5
17-10-52	70	0	5										5
17-10-68	55	65.5							1	2	1	1	5
17-10-53	68	0	5										5
17-10-75	71	57.7				1	2		2				5
17-10-72	65	1.5	2	1	2								5
17-10-48	65	0	10										10

[a] Most of the duplicated parental and F_4 plots contained from 65 to 70 plants. Thirty-two plots of each parent were grown, thus making 16 duplicates comparable to the F_4 families.

It is obvious from Table 2 that all the F_3 families classified as low-infection strains were heterozygous, as they produced both smutted and smut-free F_4 families. On the other hand, all the families which showed a high, and some of those which showed an intermediate degree, of smut infection in F_3 produced in F_4 only families which contained smutted plants.

In Table 3 are shown the F_4 families and the parents among them grown in 1928. The F_3 lines from which these F_4 families were derived were grown in the same year (1926) as the F_3 lines reported in Table 2.

There were eight F_3 lines noted as zero infection which were grown in F_4. All but one proved to be homozygous for this character. Strain 17–10–15 produced both smutted and smut-free F_4 families,

and hence probably should have been classed as a low-infection rather than as a zero-infection strain.

TABLE 3.—*Percentage of smutted plants among the F_3 families, number of smutted plants in various percentage classes among the respective F_4 families, and the parents grown among the latter, of an oat cross, Gopher × Black Mesdag, and the reciprocal, grown in 1928.*

Parent or progeny	F_3 families		Number of plants in various smut-percentage classes among F_4 families															Total
	Plants	Smutted	0	2.5	7.5	12.5	17.5	22.5	27.5	32.5	37.5	42.5	47.5	52.5	57.5	62.5	67.5	
	Number	*Per cent*																
Gopher				3	3	3												9
Black Mesdag			9															9
16–15–18	63	20.6	2	1	1	1												5
16–15–3	60	0	5															5
16–15–51	65	30.8							1			2		1	1			5
16–15–29	64	0	5															5
16–15–74	46	33.3						1	1	1				1		1		5
16–15–41	55	0	5															5
16–15–77	66	40.9				1					1		1	2				5
16–15–49	63	0	5															5
17–10–2	67	37.3			1	1	1	1	1									5
17–10–8	69	0	5															5
17–10–10	58	36.2					1		1	2	1							5
17–10–15	62	0	3	1		1												5
17–10–17	68	22.1							1	1	2			1				5
17–10–27	65	0	5															5
17–10–43	75	42.7						1	1	1	1			1				5
17–10–47	68	0	5															5
17–10–60	72	34.7			1			1		2			1					5
17–10–63	70	38.6					1	1			1			2				5

Strain 16–15–18 with an infection of 20.6 per cent smut in F_3 produced both smutted and smut-free F_4 families, whereas strain 17–10–17, with approximately the same degree of infection (22.1 per cent) in F_3, produced nothing but rather highly susceptible F_4 lines. The remaining eight strains, which ranged in smut infection from 30.8 to 42.7 per cent in F_3, produced only F_4 families which contained some smutted plants.

From the data reported it seems reasonable to conclude that the zero-infection F_3 lines are homozygous, that the low-infection F_3 lines are for the greater part heterozygous, and that the moderately high and the highly susceptible F_3 lines are probably homozygous for at least one main factor for susceptibility.

F_5 FAMILIES

The F_5 families and the parents grown among them in 1928, together with the ancestral F_4 lines grown in 1927, are shown in Table 4. In general, the F_5 lines derived from zero-infection F_4 families bred true to this condition. The exceptions were strains 16–15–31–1 and 16–15–53–3, each of which produced one F_5 family that contained a single smutted plant.

Family 16–15–8–5 contained 10.2 per cent smutted plants in 1927. From Table 2 it may be seen that three of the sibs of this family were recorded as zero infection, and one fell in the class of 2.5 per cent infection. The five F_5 families from line 16–15–8–5 (Table 4) fell into the following classes of percentage infection: One, zero; three, 2.5; and one, 12.5.

Family 16–15–7–5 was one of four sibs which showed a low percentage of smut infection in 1927. The other related F_4 line showed a zero percentage infection. In F_5, of the five descendant lines from 16–15–7–5, but one showed any smut and that one in a single plant only. Some of these lines may have been susceptible but escaped infection or, if infected, failed to give external evidence of it. A further test of these strains will be made in the F_6 generation.

TABLE 4.—*Percentage of smutted plants among the F_4 families, number of smutted plants in various percentage classes among the respective F_5 families, and the parents grown among the latter, of an oat cross, Gopher×Black Mesdag, and the reciprocal*

Parent or progeny	F_4 families		Number of plants in various smut-percentage classes among F_5 families							Total
	Plants	Smutted	0	2.5	7.5	12.5	17.5	22.5	27.5	
	Number	*Per cent*								
Gopher	------	------	2	4	5	3	------	------	------	14
Black Mesdag P	------	------	14	------	------	------	------	------	------	14
16–15–4–1	62	0	1	------	------	------	------	------	------	1
16–15–4–2	64	0	1	------	------	------	------	------	------	1
16–15–4–4	55	0	1	------	------	------	------	------	------	1
16–15–4–5	59	0	1	------	------	------	------	------	------	1
16–15–8–1	45	0	5	------	------	------	------	------	------	5
16–15–8–5	49	10.2	1	3	------	1	------	------	------	5
16–15–7–3	33	0	5	------	------	------	------	------	------	5
16–15–7–5	59	1.7	4	1	------	------	------	------	------	5
16–15–16–2	70	0	2	------	------	------	------	------	------	2
16–15–16–4	67	0	3	------	------	------	------	------	------	3
16–15–31–1	64	0	4	1	------	------	------	------	------	5
16–15–31–5	61	1.6	2	2	1	------	------	------	------	5
16–15–1–5	55	0	5	------	------	------	------	------	------	5
16–15–35–1	54	0	3	------	------	------	------	------	------	3
16–15–17–2	63	0	5	------	------	------	------	------	------	5
16–15–53–3	69	0	2	1	------	------	------	------	------	3
16–15–35–4	59	0	3	------	------	------	------	------	------	3
16–15–68–5	51	2.0	------	2	2	1	------	------	------	5
17–10–1–1	53	0	3	------	------	------	------	------	------	3
17–10–62–1	50	0	3	------	------	------	------	------	------	3
17–10–48–1	51	0	3	------	------	------	------	------	------	3
17–10–1–2	64	0	3	------	------	------	------	------	------	3
17–10–30–1	43	2.3	5	------	------	------	------	------	------	5
17–10–53–2	45	0	3	------	------	------	------	------	------	3
17–10–48–2	55	0	3	------	------	------	------	------	------	3
17–10–1–3	64	0	3	------	------	------	------	------	------	3
17–10–62–3	55	0	3	------	------	------	------	------	------	3
17–10–48–3	50	0	3	------	------	------	------	------	------	3
17–10–1–4	65	0	3	------	------	------	------	------	------	3
17–10–53–3	58	0	3	------	------	------	------	------	------	3
17–10–1–5	61	0	3	------	------	------	------	------	------	3
17–10–53–5	60	0	3	------	------	------	------	------	------	3
17–10–48–5	57	0	3	------	------	------	------	------	------	3
17–10–48–6	46	0	2	------	------	------	------	------	------	2
17–10–48–7	38	0	2	------	------	------	------	------	------	2
17–10–48–8	54	0	2	------	------	------	------	------	------	2
17–10–48–9	54	0	2	------	------	------	------	------	------	2
17–10–48–10	55	0	2	------	------	------	------	------	------	2
16–15–17–6	51	0	4	------	------	------	------	------	------	4
17–10–68–1	46	34.8	------	1	1	------	------	------	1	3
17–10–68–5	57	38.6	------	------	1	1	------	1	------	3
17–10–64–1	50	10.0	2	------	1	------	------	------	------	3
17–10–64–2	57	30.0	------	2	------	------	1	------	------	3

Line 16–15–31–5 is one of three F_4 families with the same ancestry that showed a low percentage of smut. The other two related F_4 families showed no smut. The F_5 descendants of 16–15–31–5 showed percentages of smut as follows: Two families with zero infection; two families which fell into the 2.5 per cent class; and one family into the 7.5 per cent class. In other words, these five F_5 families which descended from 16–15–31–5 reacted to smut in a manner very similar to that of the five F_4 families from 16–15–31.

Strain 16–15–68–5 was one of five F_4 lines with the same ancestry which showed a low percentage of smut infection. It is obvious from Table 4 that the five F_5 lines descended from this strain also showed a low percentage of smut infection, indicating homozygosity for the character.

In 1927 strain 17–10–30–1 was the only one of five F_4 families from the same F_3 line which showed any smut, and this strain showed smut in but a single plant. Although precaution was taken to prevent an accidental mixture, there is always the possibility of contamination from this source. Then, too, there is an appreciable amount of natural crossing in oats at Morgantown, as has been shown in a previous publication.[6] Whatever the cause of the single apparently susceptible plant observed in the F_4 family, none of the F_5 descendants showed any smut. The evidence strongly indicates that the F_3 line 17–10–30 was homozygous for the main factor controlling smut reaction.

Three F_5 families were grown from low-infection strain 17–10–64–1. Two of these showed no evidence of smut and one showed a percentage of smut that fell into the 7.5 class. This result was hardly expected, as F_4 strain 17–10–64–1 was one of five which showed smut. A further test in the F_6 generation will be made. This is of particular importance because of the fact that two of the duplicate plots of Gopher did not show any evidence of smut infection in 1928.

The three remaining F_4 strains which showed smut in 1927 and which were tested in F_5 were classed as moderately high in susceptibility. Each one of the three produced one F_5 family which showed a degree of smut infection greater than the most susceptible duplicate plots of Gopher.

PEDIGREES OF CERTAIN F_3 LINES

In order to bring some of the pedigrees together for comparison Table 5 has been prepared. It will be observed that all of the lines listed in the table which gave no evidence of smut infection in F_3 bred true for this condition through the F_5 generation. Two of the three F_3 families which showed a relatively low smut infection produced both infected and noninfected descendants. The third family, 16–15–31, produced three infected and two noninfected F_4 lines, and of the five F_5 families grown from one of the noninfected strains four showed no evidence of smut and one showed smut in a single plant only. The data seem to indicate the possibility that 16–15–31 also was heterozygous for smut reaction. Strain 16–15–68 contained 28.8 per cent smutted plants in F_3, a percentage well within the range exhibited by the Gopher parent grown the same year. The five F_4 families likewise showed percentages of smutted plants similar to Gopher grown that year, and again in F_5 the Gopher parent and the five families descended from one of the F_4 lines showed similar reaction, thus indicating that the genotypes of these lines are similar to that of one of the parents. The highly susceptible F_3 families, 17–10–64 and 17–10–68, produced only susceptible F_4 lines. The group from the former ranged from 10.0 to 29.8 per cent of smutted plants and from the latter 29.8 to 42.9 per cent. However, in F_5 two families descended from 17–10–64 and one from 17–10–68 gave

[6] GARBER, R. J., and QUISENBERRY, K. S. NATURAL CROSSING IN OATS AT MORGANTOWN, WEST VIRGINIA. Jour. Amer. Soc. Agron. 19: 191–197. 1927.

no evidence of infection. This fact may or may not be of genetic significance, particularly in view of the somewhat erratic smut epidemic obtained among the F_5 families. Further tests are being made. With the technic used in this experiment considerable variation in the percentages of smutted plants among the highly susceptible line was expected, but families without any smutted plants were not expected. It is evident from Table 5 that some of the F_5 families from the two highly susceptible F_3 lines again showed transgressive susceptibility.

TABLE 5.—*Pedigrees and number of smutted plants in various percentage classes among certain F_5 lines of the oat cross, Gopher × Black Mesdag, and the reciprocal*

Progeny	Smutted in F_3	Progeny	Smutted in F_4	Number of plants in various smut-percentage classes among F_5 families						
				0	2.5	7.5	12.5	17.5	22.5	27.5
	Per cent		*Per cent*							
16-15-7	12.5	16-15-7-3	0	5						
		16-15-7-5	1.7	4	1					
16-15-8	1.5	16-15-8-1	0	5						
		16-15-8-5	10.2	1	3		1			
16-15-16	0	16-15-16-2	0	2						
		16-15-16-4	0	3						
16-15-17	0	16-15-17-2	0	5						
		16-15-17-6	0	4						
16-15-31	1.5	16-15-31-1	0	4	1					
		16-15-31-5	1.6	2	2	1				
16-15-35	0	16-15-35-1	0	3						
		16-15-35-4	0	3						
16-15-68	28.8	16-15-68-5	2.0		2	2	1			
17-10-1	0	17-10-1-1	0	3						
		17-10-1-2	0	3						
		17-10-1-3	0	3						
		17-10-1-4	0	3						
		17-10-1-5	0	3						
17-10-48	0	17-10-48-1	0	3						
		17-10-48-2	0	3						
		17-10-48-3	0	3						
		17-10-48-5	0	3						
		17-10-48-6	0	2						
		17-10-48-7	0	2						
		17-10-48-8	0	2						
		18-10-48-9	0	2						
		18-10-48-10	0	2						
17-10-53	0	17-10-53-2	0	3						
		17-10-53-3	0	3						
		17-10-53-5	0	3						
17-10-62	0	17-10-62-1	0	3						
		17-10-62-3	0	3						
17-10-64	50.7	17-10-64-1	10.0	2		1				
		17-10-64-2	30.0		2			1		
17-10-68	65.5	17-10-68-1	34.8	1		1				1
		17-10-68-5	38.6			1	1		1	

INHERITANCE OF SMUT REACTION

A considerable number of F_1 plants have been grown in connection with this investigation, but even though the crossed seed was treated with smut spores no smutted F_1 plants have been found. In 1928 some crossed seed was hulled, treated with smut, and then planted. Of the 23 plants produced, none showed any evidence of infection. Resistance apparently behaved as a dominant in the cross Gopher × Black Mesdag and the reciprocal.

In considering the inheritance of smut reaction it may be well first to reexamine the 100 F_3 families grown in 1926, when a severe

epidemic was obtained, and take into account the behavior of the F_4 generation. To aid in this reexamination Table 6 has been prepared. A family was considered as segregating if it produced both infected and noninfected descendant lines. Of course this classification does not take into account families which may be segregating for degree of susceptibility.

TABLE 6.—*Breeding behavior of certain F_3 families as revealed in the F_4 generation of an oat cross, Gopher × Black Mesdag, and the reciprocal*

Smut percentage class in F_3	Breeding nature of F_3 plants as shown in F_4	F_3 families
		Number
0	Homogynous resistant	19
	Segregating	2
0.1– 9.9	do	4
10 –19.9	do	3
20 –29.9	Homogynous susceptible	4
	Segregating	3
30	Homogynous susceptible	13

It is apparent from Table 6 that of the 21 F_3 families which showed no smut, 19 bred true to this condition in F_4. In all cases at least 5 F_4 families were grown from each F_3 line that was tested. The 2 F_3 lines which segregated in F_4 should have been classified as low-infection strains. The 7 F_3 families which showed a percentage of smut infection between 0 and 19.9 segregated in the next generation, and of the 7 F_3 lines with a percentage of smutted plants from 20 to 29.9, inclusive, 4 bred true and 3 segregated. All of the 13 F_3 lines with a percentage of smutted plants 30 or above bred true. In view of this situation, the 100 F_3 families grown in 1926 (Table 1) may be classified as follows: Nineteen bred true for zero infection; 64 segregated and 17 bred true for more or less infection, a ratio which departs rather significantly from monohybrid expectation (P = approximately 0.03 with 2 degrees of freedom [7]). If the segregating families and those which bred true for more or less infection are grouped together, the deviation (6.0 ± 2.9) is hardly significant.

It will be observed from Table 6 that only seven F_3 families which showed percentages of smutted plants between 0.1 and 19.9 were tested in F_4. It is possible that if more families in this F_3 group had been grown, a few homozygous ones would have been found. Moreover, it should be pointed out that a few F_2 plants, from which the F_3 lines were derived, were eliminated because they were infected with smut and produced little if any seed. One or both of these causes may have been instrumental in producing the departure from expectation noted above.

Considering the 50 F_3 families grown in triplicate plots in 1928 (Table 1) and placing them in two classes, those which showed no smutted plants and those which did, a ratio of 13 to 37, respectively, is obtained; a very close agreement with a 1:3 ratio. It seems reasonable to conclude that one main factor difference was operative in determining the resistance or immunity of the Black Mesdag parent in this cross.

[7] FISHER, R. A. STATISTICAL METHODS FOR RESEARCH WORKERS. 239 p., illus. London. 1925.

The data collected from the F_5 generation for the most part bore out the conclusion just stated. Some irregularities did appear, but they may have been due to nongenetic causes. Further tests are being made in the F_6 generation.

TRANSGRESSIVE SEGREGATION

Families were obtained in the F_3 and later generations which contained a distinctly greater percentage of smutted plants than did the susceptible parent grown in close proximity, thus indicating that transgressive segregation had taken place. It seems probable that the Black Mesdag parent brought into the cross one or more factors which increased susceptibility when associated with the susceptibility factor of Gopher. The results obtained thus far may be explained fairly satisfactorily by assuming that Black Mesdag carries a dominant factor (*R*) which gives high resistance, if not actual immunity, and another factor (*i*) which when associated with the homozygous condition for susceptibility (*r*) of the Gopher parent results in transgressive segregation. The reaction of Gopher to smut suggests that the *I* factor carried by it may act as an inhibitor to *r* or that it is an additional factor for resistance but less potent than the *R* factor carried by Black Mesdag.

A tentative factorial analysis follows:

Parents:	F_2	F_3
Black Mesdag, *R R i i*.	*R R I I*	
Gopher, *r r I I*.	2 *R R I i*	Breed true for resistance.
	R R i i	
	2 *R r I I*	
	4 *R r I i*	Low susceptibility and segregate in F_4.
	2 *R r i i*	
	r r I I	Breed true for susceptibility of Gopher.
	2 *r r I i*	Somewhat more susceptible than Gopher.
	r r i i	Breed true for high susceptibility.

On the basis of this hypothesis approximately one-sixteenth, or 9.4, of the 150 F_3 families would be expected to show transgressive susceptibility. There were 13 such families actually obtained, i. e., families which showed percentages of smutted plants greater than the most susceptible plots of the Gopher parent. The departure from expectation is 3.6 ± 2.

The F_3 families homozygous for *R* (should) breed true for zero infection, and for the most part such results were actually obtained. The F_3 families from F_2 plants heterozygous for *R* (should) show low susceptibility similar to Gopher, but (should) show segregation in the F_4 generation. The seven F_3 families which showed a low infection and the two additional F_3 families which did not show any smutted plants, but probably should have been classified as low-infection strains, all segregated in F_4. Of the seven F_3 families which showed percentages of smutted plants from 20 to 29.9, inclusive, four segregated and three showed some smutted plants, but only one (16–15–68) of this latter group apparently bred true for the degree of susceptibility of the Gopher parent. On the basis of the above hypothesis about one-ninth of the slightly susceptible F_3 strains would be expected to breed true.

Mention already has been made of the fact that the black-color gene *B* of the Mesdag parent seems to be linked with the *i* factor. Certain back crosses have been made, and it is hoped that the data obtained from these will not only clear up this linkage relation, but will also clarify the inheritance situation.

SUMMARY AND CONCLUSIONS

Gopher, a pure-line selection of oats moderately susceptible to smut, was crossed with a highly resistant if not immune pure-line selection of Black Mesdag. The inheritance of resistance to smut in relation to seed color has been studied through the F_5 generation.

Resistance to smut is apparently dominant and controlled by a single main factor difference. Evidence of a modifying factor or group of factors was also obtained. Certain lines descendant from the cross showed distinctly greater susceptibility to smut than did the Gopher parent grown at the same time.

There is apparently a linkage between the black-color gene and a modifying factor which is bringing about transgressive segregation for susceptibility.

THE VITAMIN-C CONTENT OF FRESH SAUERKRAUT AND SAUERKRAUT JUICE[1]

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INTRODUCTION

There is a considerable amount of advertising before the public proclaiming the value of sauerkraut and sauerkraut juice as sources of the vitamins. Statements such as "contains all the vitamins" and "rich in vitamins A, B, C, and D" have been used, but these statements appear to be based upon general observations rather than upon experimental evidence. In some cases it is said that cabbage contains all the vitamins, and the inference is drawn that sauerkraut likewise contains all the vitamins. In view of the well-known instability of vitamin C, it appears legitimate to raise the question whether this vitamin is present in a product which has undergone such operations as are involved in the making and marketing of sauerkraut.

Some justification for the belief that sauerkraut must contain vitamin C is found in old reports regarding its use as an antiscorbutic agent on long sea voyages and the freedom from scurvy in certain regions where sauerkraut was extensively used as a food (*4, p. 45*).[2] But many other and often unobserved factors operate in such cases, and it is unsafe to place much reliance on these old reports.

A review of the literature seems to show only two pieces of experimental work on the vitamin-C content of sauerkraut. Ellis, Steenbock, and Hart (*3*), in their study of the stability of the antiscorbutic vitamin and its response to various treatments, include data on raw sauerkraut obtained from a local grocery. In this work they fed the sauerkraut at 2.5 and 5 gm. levels (daily) to guinea pigs on a scorbutic ration. On the 2.5-gm. level the animals died of scurvy within four to five weeks, while those on the 5-gm. level died in about six weeks. The conclusion of Ellis and his associates was that at these levels the sauerkraut showed no evidence of possessing antiscorbutic properties.

In a later paper, Wedgewood and Ford (*9*) report that sauerkraut juice in quantities ranging from 0.5 gm. to 5 c. c. per day did not prevent scurvy in guinea pigs. They are not very explicit as to how the sauerkraut was made, and it is therefore difficult to decide whether or not their sauerkraut can be taken as representative of the commercial product. Their work is also open to the criticism that the sauerkraut juice used may have lost its potency as a result of storage. The juice for the entire feeding period (26 to 33 days) was expressed from the sauerkraut at the beginning of the experiment and kept on

[1] Received for publication Apr. 13, 1929; issued December, 1929. Published with the permission of the director of the Wisconsin Agricultural Experiment Station.

[2] Reference is made by number (italic) to "Literature cited," p. 971.

ice until it was fed. Since the juice was not kept under anaerobic conditions, it is probable that, even if present at the beginning of the feeding period, vitamin C would have disappeared from the juice in the course of the experiment.

In view of the lack of experimental evidence available, it seemed desirable to make a study of the vitamin-C content of sauerkraut.

EXPERIMENTAL WORK

Two rations were used in this work. The first is a modification of that employed by Cohen and Mendel (*1*) and also by Parsons and Reynolds (*6*). The ration as given below has been used in this laboratory for the past three years and is called the soybean scurvy ration. Its composition is as follows: Soybean flour, 1,620 gm.; dried yeast, 120 gm.; purified casein, 105 gm.; calcium carbonate, 60 gm.; filter paper, 40 gm.; butterfat, 100 gm. The soybean flour was a commercial product the preparation of which involves heating. The yeast also was a commercial product and was a reliable source of the vitamin-B complex. The casein was purified by soaking for a week in water slightly acidified with glacial acetic acid (about 5 c. c. per 6 quarts of tap water). This water was changed every day. The filter paper was cut into small pieces and beaten in distilled water until a fine pulp was obtained. This was poured upon the mixed dry ingredients and the mass was rubbed until evenly mixed. When dry, it was ground and mixed thoroughly with melted butterfat.

The second ration is that used by Ellis, Steenbock, and Hart (*3*). It is called the alfalfa scurvy ration and contains the following: Alfalfa (autoclaved 30 minutes at 15 pounds pressure), 25 per cent; rolled oats, 69 per cent; purified casein, 5 per cent.

Animals that were used as positive or negative controls were given the above rations with the addition of 60 gm. of sodium chloride to the soybean ration and 1 per cent sodium chloride to the alfalfa ration. Animals fed sauerkraut or sauerkraut juice received their sodium chloride from these materials.

In the early part of the work both rations were used, but since the two rations were found to be quite similar from the standpoint of production of scurvy, only the alfalfa ration was used in the later experiments.

The protection of guinea pigs from scurvy was the method used in the investigation. After the preliminary feeding period described below, cabbage was removed from the diet and sauerkraut only was fed with the basal ration for a period of 60 to 75 days or until the animal died. The onset of scurvy symptoms, swollen wrists or a "jerky run," was noted and autopsy performed at the end of the experiment or after death.

On autopsy, the following signs of scurvy were looked for: Hemorrhages and swelling of the wrists or elbows of the fore legs and also below the hip joint of the hind legs; hemorrhages or bleeding at the costochondral juncture of the ribs, loose teeth, and any possible hemorrhages in the abdominal organs or in the peritoneum. The condition of the residue in the intestinal tract was also noted.

The weight of the guinea pigs when the cabbage was removed from the diet and sauerkraut feeding was begun varied from 211 to 276 gm., the average being about 240 gm. Young guinea pigs when received in

the laboratory or when taken from the mother were placed on the scurvy ration plus cabbage and allowed to eat ad libitum. With the first group of guinea pigs there was no preliminary feeding of sauerkraut before the experiment was begun, and difficulty was experienced in getting some of the animals to eat their portions. With all the other groups the following procedure was used: On the first day the basal ration plus cabbage only was fed; on the second day the cabbage was ground in a food chopper, a small amount (5 to 10 gm.) of sauerkraut was added to the cabbage, and the mixture was put into the basal ration. The sauerkraut was increased each day, but at no time was the amount of cabbage in the mixture less than 5 gm. per guinea pig in the group. In this way the guinea pigs gradually became accustomed to the taste of the sauerkraut, and as a general rule there was no trouble in getting them to eat it. When the experiment was begun each animal was placed in a separate cage, and the dose of fresh sauerkraut was mixed with the ration. The fresh sauerkraut juice was given to the animals by means of a medicine dripper.

The sauerkraut used in this experiment was made from All Seasons variety of cabbage, cut during the first week in November, 1927, and stored outside in a cool place for a week before it was made into sauerkraut. Three hundred pounds of shredded cabbage were mixed with 7½ pounds of salt and packed into 45-gallon barrels. A loose-fitting cover held down by a heavy weight was placed on top of the cabbage. These barrels of cabbage were allowed to ferment for 90 days at a temperature which ranged from 60° to 65° F. After this time the barrels were opened and the top sauerkraut, to the depth of 1 foot, was discarded. The sauerkraut was of good quality and possessed an acidity of 1.7 per cent calculated as lactic acid. Each day fresh samples were removed from points well below the surface, thus reducing or eliminating the factor of oxidation. The sauerkraut was ground in a food chopper and fed to the animals within as short a time as possible. The sauerkraut juice was obtained by putting the sauerkraut in a fine-meshed canvas bag and squeezing out the juice.

The study was made on three levels: Group 1 on 10 gm. of sauerkraut or sauerkraut juice daily; Group 2 on 5 gm. of sauerkraut daily; and Group 3 on 2.5 gm. of sauerkraut daily.

In the first group, three guinea pigs that failed to eat their portions were given partly neutralized sauerkraut in order to stimulate consumption. After the acidity of the sauerkraut had been determined, each day's sample was half neutralized with N/1 NaOH immediately before feeding. These animals (Nos. 4, 5, and 15) seemed to eat the partly neutralized sauerkraut better than the unneutralized. They were fed this type of sauerkraut throughout the 60 days of the experiment.

DISCUSSION OF RESULTS

HIGH LEVEL OF SAUERKRAUT AND SAUERKRAUT JUICE (10 gm.)

The first group of guinea pigs were fed 10 gm. daily of sauerkraut or sauerkraut juice. Inasmuch as Ellis, Steenbock, and Hart (*3*) reported no antiscorbutic value at 2.5 and 5 gm. levels, it seemed advisable to begin with the higher level in order to insure the detection of any vitamin C if it were present.

The individual weight curves (figs. 1 and 2) show that a 10-gm. level of this sauerkraut or the juice therefrom gave good growth and

complete protection from scurvy. The daily gain in weight of the 7 animals which received juice ranged from 2.26 to 5.33 gm. and averaged 3.83 gm. The figures for the 8 animals fed sauerkraut

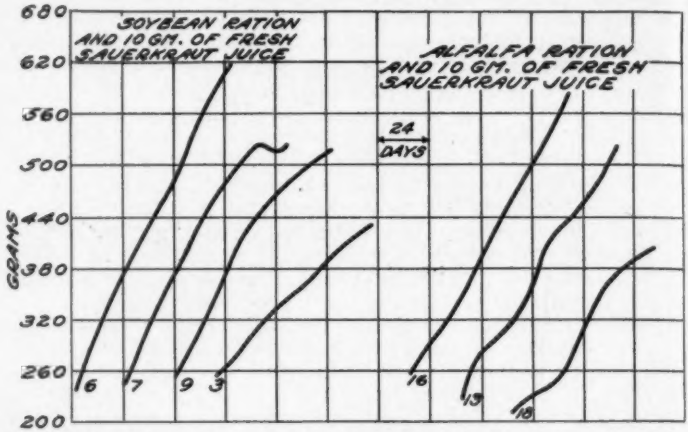

FIGURE 1.—Growth curves showing the antiscorbutic effect of 10 gm. of fresh sauerkraut juice daily when added to two scurvy rations fed to guinea pigs; autopsies at the end of 75 days disclosed no evidences of scurvy in any of the animals

ranged from 2.22 to 4.85 gm. and averaged 3.53 gm. The 2 animals which received 5 gm. of raw cabbage daily (fig. 4) made daily gains of 4.06 and 4.90 gm., respectively, and averaged 4.48 gm. Of the 15 animals fed sauerkraut or its juice at this level 6 grew faster than 1 of the animals (No. 11) that received 5 gm. of cabbage, and 3 made greater

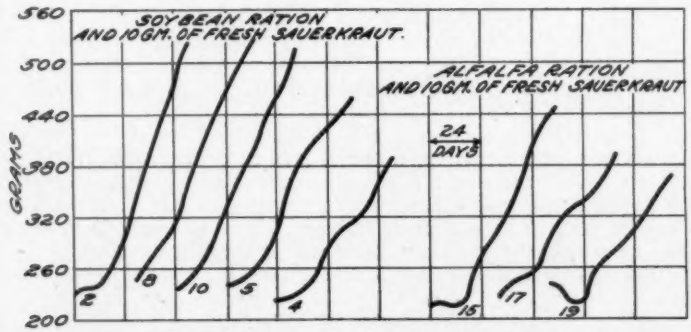

FIGURE 2.—Growth curves showing the antiscorbutic effect of 10 gm. of fresh sauerkraut daily when added to two scurvy rations fed to guinea pigs; autopsies at the end of 60 days disclosed no evidences of scurvy in any of the animals

daily gains than either of the positive controls. Because of the limited number of animals used as cabbage controls, too much emphasis should not be placed on the comparison, but it appears that 10 gm. of sauerkraut or sauerkraut juice contain about the same amount of vitamin C as 5 gm. of raw stored cabbage.

MEDIUM AND LOW LEVELS OF SAUERKRAUT (5 and 2.5 gm.)

When, after six to seven weeks, it was evident that the 10-gm. levels of sauerkraut and sauerkraut juice were each giving protection from scurvy and allowing good growth, feedings at 5 and 2.5 gm. levels

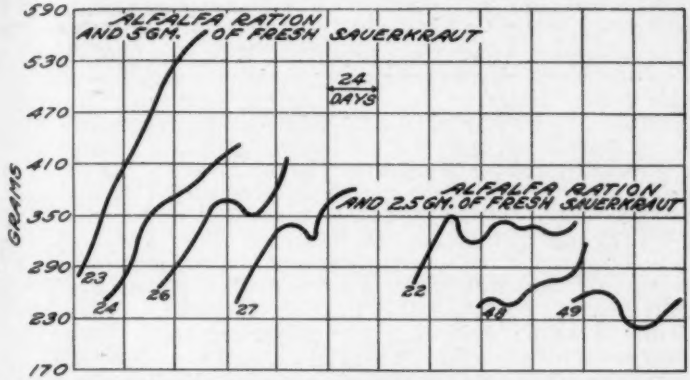

FIGURE 3.—Growth curves showing the antiscorbutic effect of 5 and 2.5 gm. of fresh sauerkraut daily when added to the alfalfa scurvy ration fed to guinea pigs. Autopsies performed at the end of 60 days on the animals fed at the 5-gm. level disclosed no evidences of scurvy in any case. An autopsy on animal No. 22 at the end of 75 days showed brittle leg bones; that on No. 48 at the end of 55 days, some swelling and muscular hemorrhage; that on No. 49, a slight swelling on one leg. These last three animals received but 2.5 gm. of fresh sauerkraut daily

were begun. Although growth on a 5-gm. level was not so consistently good as on the 10-gm. level, there was no evidence of scurvy. The daily gain in weight of the four guinea pigs receiving 5 gm. of

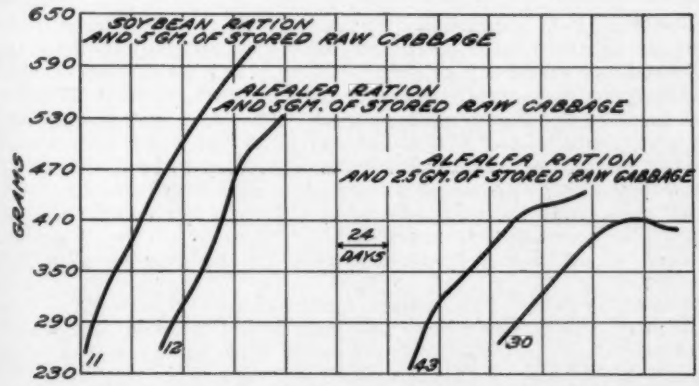

FIGURE 4.—Growth curves showing the antiscorbutic effect of 5 and 2.5 gm. of stored raw cabbage daily when added to two scurvy rations fed to guinea pigs

sauerkraut ranged from 2.33 to 4.97 gm. and averaged 3.30 gm. (Fig. 3.) This average gain is slightly lower than that for the animals on 10 gm. of juice or sauerkraut, but two of the guinea pigs, Nos. 23 and 24, have weight curves which are similar to many of the weight

curves of animals receiving 10 gm. of sauerkraut. It seems evident that a 5-gm. level of this sauerkraut was well within the protection level and also allowed good growth.

The growth on the 5-gm. level of sauerkraut was better than that obtained from one-half this quantity of cabbage. The gain per day on 2.5 gm. of cabbage (Fig. 4) was 2.92 gm. as compared with 3.30 on 5 gm. of sauerkraut. As already pointed out in the comparison of the higher levels of sauerkraut and of cabbage, the quantity of vitamin C in the sauerkraut was approximately equal to one-half of that contained in the cabbage.

Six animals were started on a 2.5-gm. level of sauerkraut, but only three of them lived through the experimental period. Three guinea pigs grew fairly well for 16 to 36 days, but at the end of this time there was a sudden drop in weight which was accompanied by a refusal to eat. The autopsy showed abnormal intestinal conditions. Guinea pig No. 22 was chloroformed at the end of 75 days instead of the usual 60 because of the several periods of loss and gain in weight of this animal. Although there were numerous fluctuations in the weight curve, it is evident that from the twentieth to the seventy-fifth day the animal was merely maintaining its weight. On autopsy, the leg bones were found to be very brittle and were easily fractured. Although there were no signs of hemorrhage, the brittle leg bones may have been due to a latent condition of scurvy.

Guinea pigs Nos. 48 and 49, also receiving 2.5 gm. of sauerkraut but started a month later than any of the other animals, were chloroformed at the end of 55 days because the sauerkraut was so nearly gone that satisfactory samples below the surface could not be obtained. Again, the weight curves (fig. 3) seem to indicate that 2.5 gm. of sauerkraut are needed for a maintenance level. Autopsy showed that with both animals there was slight evidence of scurvy.

Eddy et al. (*2*) consider 1 gm. of raw cabbage as the minimum protective dose. Unpublished data from this laboratory obtained by DeVilbiss showed that 1 gm. of raw cabbage added daily to Sherman's (*8*) basal diet would support life for 90 days, but the autopsy revealed brittle bones and teeth. In the present sauerkraut study 2.5 gm. of raw cabbage was the lowest level used. This level gave good growth for 44 to 58 days, and after that time the weight curve flattened off. (Fig. 4.) If 1 gm. of raw cabbage is considered to be the minimum protective level, with perhaps slight signs of scurvy, and 2.5 gm. of fresh sauerkraut the minimum level although not completely protective, it appears that approximately one-half of the vitamin-C content of cabbage may be destroyed in the process of making sauerkraut.

The experiments of Ellis, Hart, and Steenbock (*3*) showed a much greater destruction of vitamin C than is indicated in the present studies. These authors, however, used bulk sauerkraut obtained from a grocery store. It is probable that much of the vitamin-C content was lost after the sauerkraut was taken from the fermentation vat and repacked. As it frequently takes several weeks for a grocer to market a barrel of sauerkraut, it is not improbable that the vitamin-C content of the sauerkraut was lost by exposure to the air during the period of retailing.

That oxidation and not fermentation is the important factor in the destruction of vitamin C was shown later in the same laboratory

by Lepkovsky, Hart, Hastings, and Frazier (*5*). Sterile orange juice and tomato juice kept in test tubes plugged with cotton showed marked deterioration in 7 and 24 days, respectively. If air was excluded by means of vaseline plugs the juices retained their potency. Likewise, if the tomato juice was sealed with vaseline plugs and fermented with bacteria, some of which are characteristic of sauerkraut, the juice was still effective in the cure of scurvy. An earlier paper by Zilva (*10*) showed that the sugars present in lemon juice could be removed by yeast without appreciably altering the vitamin-C content of the juice.

It is not improbable that the strictly fermentation processes which take place in the formation of sauerkraut tend to prevent the destruction of vitamin C. In a recent paper Pruess, Peterson, and Fred (*7*) report that the gases formed in the fermentation are nearly 100 per cent carbon dioxide. The oxygen incorporated in the shredded cabbage at the time it is placed in the vat is probably speedily consumed by the respiration of the plant cells or removed from the mass by the carbon dioxide formed by the plant cells and bacteria. However, a trace of oxygen seems to persist in the sauerkraut throughout the fermentation and the partial destruction of vitamin C is probably due to this rather than to the specific action of the microorganisms.

The authors wish to point out specifically that the presence of vitamin C in the sauerkraut used in the present study does not warrant the assumption that sauerkraut as it reaches the consumer contains vitamin C. Indeed, the experiments of Ellis, Hart, and Steenbock (*3*) show that in some instances it does not. Whether or not canned sauerkraut or commercial sauerkraut juice contains vitamin C can be decided only by carefully controlled experiments that deal directly with these products.

PRESENCE OF VITAMIN C DEMONSTRATED BY RECOVERY TYPE OF EXPERIMENT

Two guinea pigs in the "negative" group, Nos. 32 and 67, were given 5 c. c. of sauerkraut juice at the onset of definite scurvy symptoms. (Fig. 5.) The juice was fed for 16 days and autopsies were performed on the animals on the seventeenth day. The autopsies indicated in both cases that the animals were practically cured of scurvy. Although the protection method was used primarily in this study, the above-mentioned data on the recovery of two of the "negative controls" strengthen the conclusion that an appreciable quantity of vitamin C was present in this sauerkraut juice.

SUMMARY

This study shows the vitamin C content of fresh raw sauerkraut and sauerkraut juice, i. e., material which was fed immediately after being taken from the barrel in which it was fermented. The sauerkraut was fed at levels of 10, 5, and 2.5 gm. daily for each guinea pig for approximately 60 days, and sauerkraut juice was fed at a level of 10 gm. for 75 days. Positive control animals were fed at 5 and 2.5 gm. levels of raw stored cabbage.

The 10-gm. level of either fresh sauerkraut or fresh juice protected from scurvy and allowed good and in some cases excellent growth. The growth curves are comparable to those of guinea pigs receiving

5 gm. of raw stored cabbage. The 5-gm. dosage of fresh sauerkraut was well within the protection level and also allowed good growth. The 2.5 gm. level of fresh sauerkraut was probably not quite enough to protect from scurvy, although a maintenance of weight was obtained. The 2.5 gm. level of raw stored cabbage allowed good growth for 44 to 58 days, and after that time the weight curve

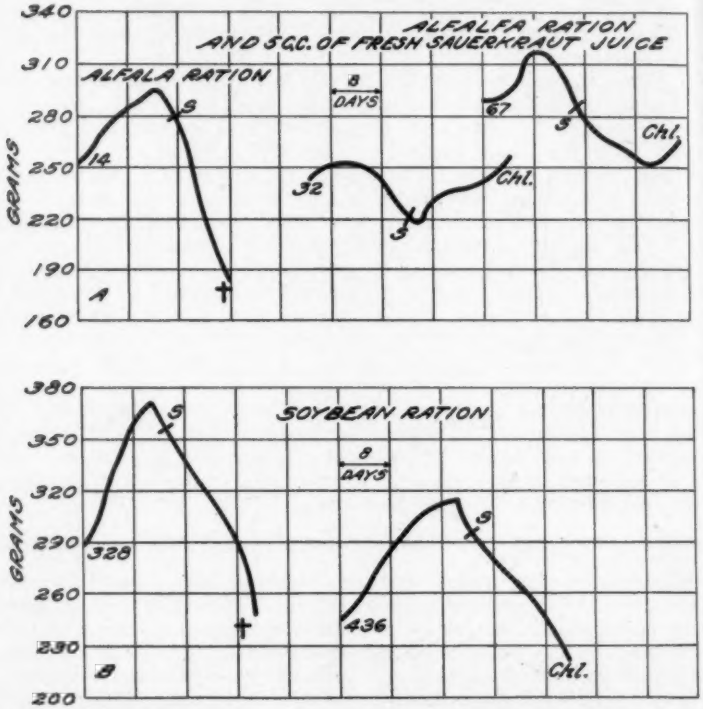

FIGURE 5.—Growth curves of guinea pigs receiving only the basal scurvy-producing rations. Definite symptoms of scurvy were present at points marked "8"; + indicates death and Chl. indicates that the animal was chloroformed. A, These animals were on the alfalfa scurvy ration. Nos. 32 and 67 were given a recovery dosage of 5 c. c. of fresh sauerkraut juice daily; autopsy at the end of a recovery period of 16 days disclosed that recovery was complete. Animal No. 14 was a "negative control" receiving no addition to the basal diet and showed, on autopsy, characteristic evidences of scurvy. B, These animals were on the soybean scurvy ration and, at autopsy, showed the characteristic evidences of scurvy

"flattened off," although there was no evidence of scurvy. A loss of vitamin C in the formation of sauerkraut is thus definitely demonstrated. This loss is approximately one-half of the vitamin-C content of cabbage.

Guinea pigs which had developed definite symptoms of scurvy recovered when fed 5 c. c. of fresh sauerkraut juice daily.

No conclusions as to the probable vitamin-C content of commercial sauerkraut are warranted by these experiments.

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